5' variants of glucocorticoid receptor mRNA: further studies of tissue-specificity and regulation.

Alistair Iain Freeman BVM&S CertSAS MRCVS

Doctor of Philosophy
University of Edinburgh
2002

VET. SCHOOL LIBR. EDINBURGH

Acknowledgements

First I must thank my supervisor Karen for her constant encouragement and help. I'm sure I wasn't her easiest PhD student, but she was endlessly patient with me. I'd also like to thank my other supervisor, Jonathan, for his advice and encouragement and the Wellcome Trust for their financial support.

Many people in the lab have helped me a lot during my PhD. Thanks to Val especially for her advice and help with cloning, sequencing, etc. etc. Thanks also to Karen and Lynne for advice about *in situ* hybridisation and for the ordering! June also deserves a big "thankyou" for assistance with the animal work and that damn grain counting system. Thanks too to Keith and the other animal unit staff. Thanks Rosemary and Elaine for sorting out loads of minor hassles with my grant and sorting out conferences etc., much appreciated. My office mates Val, Helen and Dave B: what can I say? Thanks for putting up with me and the yucca; you were great company (despite the Jethro Tull, Dave). The bonsai is still alive, by the way.... Some other people deserve a special mention. Mark, for being a mate for more years than I really want to count (and for the rats!). Jim, for the beer, Starcraft and occasionally some scientific advice too. Nick, for advice and being a generally nice bloke. Dmayo(bay 9) for the biking and the Spanish fiestas: I know nothing, amigo! Thanks too to the rest of the lab, it was a great working atmosphere. Great cake too.

I owe thanks to loads of people outside the lab as well. Thanks to Ian Cameron and my other friends at tai chi, for teaching me a lot more than a martial art. To my "brother" Dave R, for always being there. Thanks to Mum and Dad, to whom I owe a debt I can never repay. Finally, to Joan. Without you it wouldn't mean anything.

Declaration

I declare that this thesis and the work presented in it are entirely the result of my own independent investigation, except where stated in the text. This work has not been, and is not currently, submitted for any other degree.

Alistair Freeman BVM&S CertSAS MRCVS

Table of Contents

List of figures

List of Tables

List of Abbreviations

5,7-DHT	5,7-dihydroxytryptamine
5-HT	5-hydroxytryptamine
5'-RACE	5' rapid amplification of cDNA ends
11β-HSD1/2	11β-hydroxysteroid dehydrogenase type 1/2
ACTH	Adrenocorticotrophic hormone
ACTR	Acetyltransferase nuclear receptor coactivator
Ada2	Adenosine deaminase 2
Adx	Adrenalectomised
AF1/2	Activation function 1/2
AMP	Adenosine monophosphate
AP1/2	Activator protein 1/2
APRT	Adenine phosphoribosyl transferase
AR	Androgen receptor
ARA	Androgen receptor associated protein
AS	Antisense
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BST	Bed nucleus of the stria terminalis
CA	Cornu ammonis
CBG	Corticosteroid-binding globulin
cAMP	Cyclic AMP
CBP	CREB-binding protein

cDNA	Complementary DNA
C/EBP	CAAT/enhancer binding protein
CNS	Central nervous system
CREB	cAMP response element-binding protein
CREM	cAMP response element
CRH	Corticotrophin-releasing hormone
cRNA	Complementary RNA
DBD	DNA binding domain
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DP	Double positive
DRIP	Vitamin D3 receptor interacting protein
FTOC	Foetal thymus organ culture
GABA	Gamma-amino butyric acid
GATA1	Homo sapiens GATA-binding protein 1 (globin transcription factor 1)
GNAT	Gcn5-related N-acetyltransferase
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRIP	Glucocorticoid receptor-interacting protein
HAT	Histone acetyltransferase
HMG CoA	3-Hydroxy-3-Methyl-Glutaryl Coenzyme A
HNF	Hepatocyte nuclear factor
HOX	Homeobox

HPA	Hypothalamic-pituitary-adrenal
HSD	Honest significant difference
HSF	Heat shock factor
Hsp	Heat shock protein
IκB	Inhibitory κB
IGF	Insulin-like growth factor
LBD	Ligand binding domain
LT	Long term
MeCP	Methyl CpG binding protein
MEK	MAP (Mitogen-activated protein)/ERK (extracellular signal regulated kinases) kinase
MHC	Major histocompatibility factor
MMTV(LTR)	Mouse mammary tumour virus (long terminal repeat)
MOPS	3-(N-Morpholino)propanesulfonic acid
MpFC	Medial prefrontal cortex
mRNA	Messenger RNA
MR	Mineralocorticoid receptor
myc	Oncogene of the MC29 avian myelocytomatosis virus
MYST	Protein of the MOZ, YBF2/SAS3, SAS2, and Tip60 family
NAD	Nicotinamide adenine dinucleotide
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
nGRE	Negative GRE
NF-1	Nuclear factor 1
NF-κB	Nuclear factor κB
NMDA	N-methyl-D-aspartate
OCT-1	Octamer-binding protein 1

OTF	Octamer transcription factor
p/CAF	p300/CBP-associating factor
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PNMT	Phenyethanolamine-N-methyl-transferase
PolII	RNA polymerase II
POMC	Propionomelanocortin
PP	Periportal
PR	Progesterone receptor
PV	Perivenous
RAP	Receptor-associating protein
RNA	Ribonucleic acid
RPA	RNase protection analysis
RT-PCR	Reverse transcriptase PCR
S	Sense
SEM	Standard error of the mean
SP1	Selective promoter factor 1
SRC-1	Steroid receptor coactivator-1
SSC	Saline-sodium citrate
ST	Short term
STAT	Signal transducer and activator of transcription
SWI/SNF	Switch/sucrose-non-fermentable
TAT	Tyrosine aminotransferase
TBE	TRIS boric acid EDTA

TBP	TATA binding protein
TCR	T cell receptor
TEMED	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
TFIID	Transcription factor II D
TIF	Transcription intermediary factor
TSG	Tumour susceptibility gene
YAC	Yeast artificial chromosome

Publications from this thesis

Abstracts

Freeman A.I., Lyons V, Whiteley L, Seckl J.R., Chapman K.E. (2000) Region-specific expression of mRNAs encoding alternate exons 1 of the glucocorticoid receptor gene. European Journal Of Neuroscience 12: 10-10, Suppl.

Freeman A.I., Lyons V., Seckl J.R. and Chapman K.E. (2001) Tissue-specific differential expression of variant exons 1 of the glucocorticoid receptor gene. Endocrine Abstracts Vol. 1, P193

Freeman A.I., Cleasby M.E., Lyons V, Seckl J.R and Chapman K.E. (2001) Differential regulation and distribution of variant glucocorticoid receptor mRNAs. Abstracts of the 83rd Meeting of the American Endocrine Society, P1-6.

Abstract

Glucocorticoids have diverse physiological functions: they affect central nervous system function, intermediary metabolism and restore homeostasis after stress. Secretion of glucocorticoids is regulated by the hypothalamic-pituitary-adrenal (HPA) axis; negative feedback at the hypothalamus and pituitary suppresses glucocorticoid secretion while the hippocampus exerts additional control over HPA axis activity.

Glucocorticoids exert most of their actions, including negative feedback, via the glucocorticoid receptor (GR). The glucocorticoid sensitivity of a given cell/tissue is dependent on the level of GR expression. The regulation of the GR gene is complex; GR levels in adult animals are subject to glucocorticoid regulation and can be permanently "programmed" by early life events, with hippocampal GR permanently increased by neonatal handling (via alterations in serotonin turnover) and decreased by prenatal dexamethasone exposure. Evidence suggests that these effects may be mediated through differential regulation of variant exon-1 containing GR mRNAs; in rats the GR gene contains 8 protein-coding exons (exons 2-9) and at least 11 alternate untranslated exons 1 (exons 1_1-1_{11}) which may reflect transcription regulated by alternate promoters. The aim of this thesis was to further investigate the distribution of these variant GR transcripts and examine whether glucocorticoids themselves differentially regulate GR mRNA and its alternate exons 1 in a tissue and region-specific manner.

Tissue and region-specific differences in the expression of variant GR mRNA transcripts were found in rat and mouse. Most GR mRNA variants were ubiquitously expressed, but those containing exon 1_1 were restricted to rat thymus, liver and hippocampus and mouse spleen, while those containing exon 1_4 were absent from rat cerebral cortex and mouse lung, heart and abdominal fat. *In situ* mRNA hybridisation on rat brain showed that all the exons 1 studied showed differences in their regional

expression when compared to distribution of the total population of GR mRNAs. In contrast, in rat liver and thymus GR mRNA variants showed the same regional distribution as total GR mRNA with highest expression in periportal region of the liver and the thymic cortex.

To investigate whether glucocorticoids differentially regulate variant GR mRNAs, *in situ* mRNA hybridisation was used to investigate the expression variant exons 1 in the hippocampus of adult rats subjected to 72h (ST) or 3-week (LT) adrenalectomy with glucocorticoid replacement. Variant GR mRNAs containing exons 1_5, 1_7, 1_{10} and 1_{11} were not affected by adrenalectomy or supraphysiological glucocorticoid replacement in ST or LT animals. However, both adrenalectomy and supraphysiological glucocorticoid replacement significantly upregulated total GR mRNA in the hippocampus of ST animals while adrenalectomy significantly upregulated total GR mRNA in the hippocampus of LT animals.

In situ mRNA hybridisation and RNase protection analysis were used to investigate expression of variant GR mRNAs in the liver. Glucocorticoid manipulations did not significantly affect expression of variant GR mRNAs containing exons 1_5, 1_6, 1_{10} or 1_{11}. However, in ST adrenalectomised animals glucocorticoid replacement significantly downregulated GR mRNA levels compared to adrenalectomised animals given vehicle alone. Adrenalectomy had no effect on total GR mRNA expression in the LT animals, although in these animals the periportal:perivenous ratio of GR expression was significantly increased by adrenalectomy compared to sham. Preliminary data from DNase I hypersensitive site mapping in control animals showed an area of DNase I sensitive chromatin around the position of exon 1_{10}, present in the majority of GR mRNA in the liver.

In the thymus, although adrenalectomy with either high and low dose glucocorticoid replacement in ST animals caused a significant downregulation of GR mRNA in the cortex and medulla compared to sham, the expression of exons 1_1, 1_6 and 1_{10} of the GR gene was not significantly affected by glucocorticoid manipulations. There was no effect of glucocorticoid manipulation on GR or its variants in the LT animals.

These results demonstrate tissue-specific differences in the distribution of GR mRNA variants, suggesting that variations in promoter usage may have a role in determining the "set-point" of GR expression in different tissues. The observed tissue-specific effects of glucocorticoids on GR mRNA expression could not be accounted for by changes in expression of any of the variant GR mRNA transcripts studied. This suggests that either the expression of another variant mRNA (known or novel) is regulated by glucocorticoids or that expression of all or a subset of the variant GR mRNAs changed with a magnitude too small to be detected in this study.

1 Introduction

Glucocorticoids are steroid hormones which exert effects on virtually all tissues in the body. Their effects are mediated by an intracellular receptor, the glucocorticoid receptor (GR), the regulation of which is the subject of this thesis. The principal determinants of glucocorticoid action are the intracellular level of receptor and the circulating levels of hormone, although other factors also modulate their actions in various tissues. In this introduction I shall first describe the synthesis of glucocorticoids and briefly discuss some of the factors determining access of glucocorticoids to their receptor. I shall also discuss the structure and function of the GR protein and gene, with particular reference to the regulation of GR levels in various tissues and the role of GR in transcriptional regulation. The remainder of the chapter will deal with the diverse physiological effects of glucocorticoids and the various transgenic models of GR function.

1.1 Glucocorticoid structure and synthesis

1.1.1 Glucocorticoid structure

Glucocorticoids are steroid hormones derived from cholesterol, which contains 27 carbon atoms in a cyclopentanoperhydrophenanthrene nucleus of 4 rings (A-D) with a $\Delta 5$ double bond (Figure 1.1). All steroid hormones have an α-oriented hydrogen atom at carbon 5, giving the A and B rings a *trans* conformation and a nearly planar structure (Ganong, 1989).

The several groups of steroid hormone molecules have different numbers of carbon atoms. The C_{21} steroids (progestagens, glucocorticoids and mineralocorticoids) have a 2-carbon side-chain at carbon 17 of the D ring. The C_{19} steroids (testosterone, dihydroepiandrosterone and androstenedione – the androgens) have a keto or hydroxyl group at carbon 17 of the D ring and are mainly synthesised in the testis. The C_{18} steroids (the oestrogens) are derived from the androgens and are mainly synthesised in the ovary (Ganong, 1989).

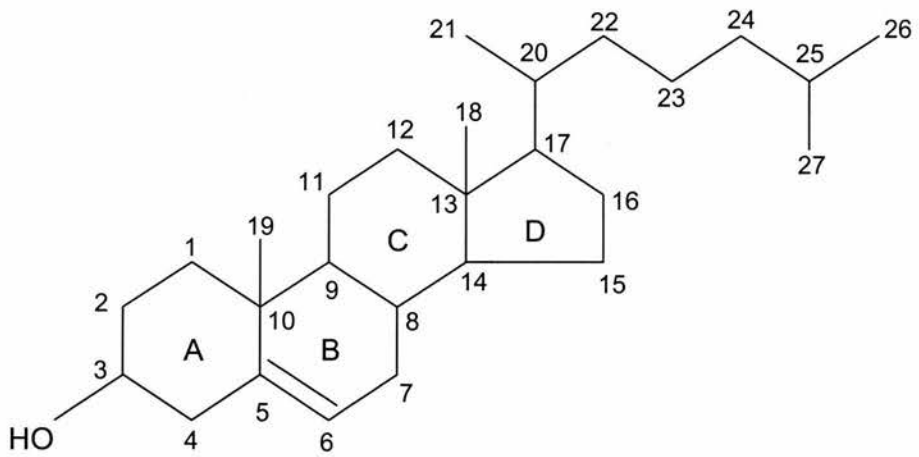


Figure 1.1: structure of cholesterol showing nomenclature of rings and numbering of carbon atoms in rings and side-chains.

1.1.2 Glucocorticoid synthesis

Glucocorticoids are synthesised in the zona fasciculata and zona reticularis of the adrenal cortex along with the mineralocorticoid aldosterone and trace amounts of oestradiol (Ganong, 1989). The synthetic pathway of glucocorticoids from cholesterol is shown in Figure 1.2. Cholesterol (supplied from circulating low-density lipoprotein or synthesised *de novo* from acetate) is esterified and stored in lipid droplets in the cytoplasm. These esters are hydrolysed to produce free cholesterol for steroid synthesis, which occurs in the mitochondria and smooth endoplasmic reticulum (Ganong, 1989). The initial step in steroid synthesis is under the control of adrenocorticotrophic hormone (section 1.9.2.2). Synthesised glucocorticoids are not stored in the adrenal but are immediately released into the circulation (Ganong, 1989). In the zona glomerulosa of the adrenal cortex, corticosterone is converted into aldosterone by aldosterone synthase (Ganong, 1989).

Rodents lack the enzyme 17α-hydroxylase, thus corticosterone is the major glucocorticoid in rodents while cortisol is the major glucocorticoid in humans and other mammals (Orth, 1998).

In addition to their synthesis in the adrenal, some evidence suggests that glucocorticoids can be synthesised locally in thymic epithelium (section 1.9.5.2). Also, the expression of mRNA for all of the enzymes required for the synthesis of adrenal steroids has now been identified in human or rat brain or cultured CNS cells (Brown et al., 2000; Gomez et al., 1996; LeGoascogne et al., 1987; Mellon and Deschepper, 1993; Yu et al., 2002; Zhou et al., 1997)), although the physiological significance of CNS glucocorticoid secretion is unknown.

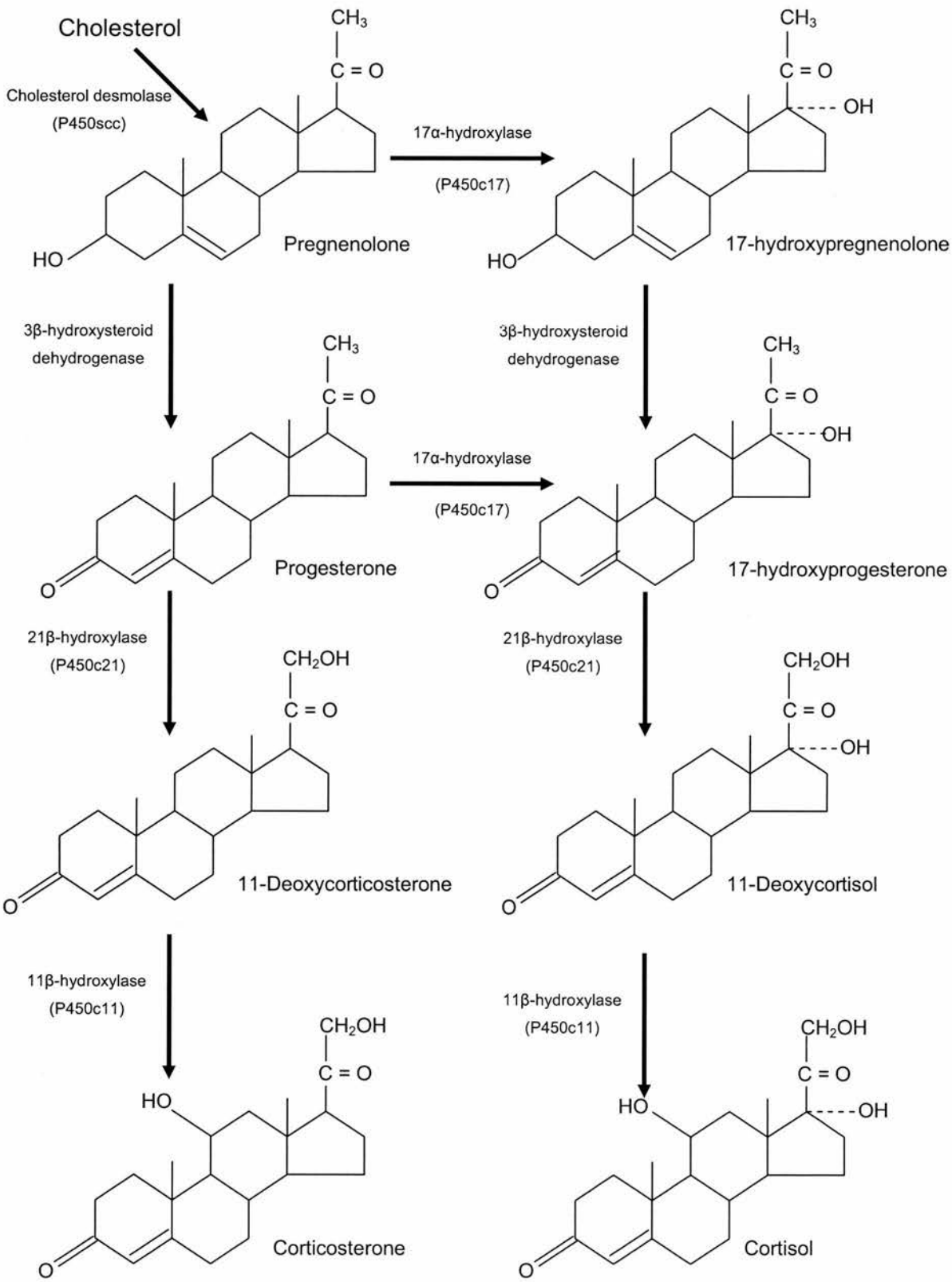


Figure 1.2: synthetic pathway of the glucocorticoid hormones. Adapted from (Ganong, 1989).

1.2 *Transport of glucocorticoids*

Approximately 96% of circulating glucocorticoids are bound to the protein corticosteroid binding globulin (CBG), with much of the remainder bound to albumin (Dunn et al., 1981). In rats, hepatic synthesis of CBG occurs in the foetus (especially around the end of the second trimester of pregnancy), is very low at birth then increases to adult levels by 3 weeks of age (Smith and Hammond, 1991).

Several factors modulate CBG levels. Adult rats show strain differences in CBG expression (Dhabhar et al., 1993) and tissue-associated CBG levels are higher in females than in males (Turner, 1992). CBG synthesis is stimulated by oestrogen, and CBG levels fall in certain disease states e.g. hepatic cirrhosis, nephrosis and multiple myeloma (Ganong, 1989). Glucocorticoids may also be important in maintaining normal CBG levels, since mice lacking a functional GR show increased circulating CBG (Cole et al., 1999).

Cortisol has a higher affinity for CBG than corticosterone, which increases its plasma half-life (60-90min compared to about 50min for corticosterone (Ganong, 1989)). Generally, if total plasma glucocorticoids exceed 20μg/dl CBG is saturated, so additional glucocorticoids circulate unbound (Ganong, 1989). Although it is generally accepted that CBG acts as a "sink", there is evidence to suggest that CBG may specifically deliver glucocorticoids to tissues (reviewed in (Pardridge, 1987)). Proteolytic cleavage results in dissociation of glucocorticoids from CBG and the release of glucocorticoids in close proximity to the target cell (Hammond et al., 1990; Pemberton et al., 1988). After delivery to target tissues, the lipid-soluble glucocorticoids enter cells mainly by diffusion across the plasma-membrane, although some active transport may occur (Orth, 1998).

Since CBG modulates delivery of glucocorticoids to tissues, the availability of glucocorticoids is determined at least partially by CBG levels. Interestingly, intracellular CBG may limit the access of glucocorticoids to their receptors e.g. in the pituitary, which has high levels of CBG (McEwen et al., 1986).

1.3 Metabolism and excretion of glucocorticoids

An important metabolism of glucocorticoids is carried out by the enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) (reviewed in (Krozowski et al., 1999; Seckl and Chapman, 1997)). 2 isozymes have been described, both members of a member of the short chain alcohol dehydrogenase/reductase family: the type II enzyme (11β-HSD2) is NAD-dependent and acts unidirectionally to convert cortisol (in humans) and corticosterone (in rats) into their inactive 11-keto metabolites, cortisone and 11-dehydrocorticosterone. The type I enzyme (11β-HSD1) is a bidirectional enzyme *in vitro* but in intact cells acts predominantly as a NADPH-dependent reductase, regenerating active glucocorticoids from cortisone or 11-dehydrocorticosterone (reviewed in (Krozowski et al., 1999; Seckl and Chapman, 1997)).

The highest 11β-HSD1 activity is found in the liver, where it may promote the effects of glucocorticoids by increasing the level of active hormone in the tissues (reviewed in (Krozowski et al., 1999; Seckl and Chapman, 1997)). Indeed, transgenic mice lacking 11β-HSD1 show reduced activation of hepatic gluconeogenic enzymes (attributed to intrahepatic glucocorticoid deficiency) and resist hyperglycaemia in stress or obesity (Kotelevtsev et al., 1997).

11β-HSD1 is widely expressed in the rat brain, with high levels in hippocampus (where glucocorticoids increase 11β-HSD1 expression (Low et al., 1994), cerebral cortex and pituitary and lower levels in hypothalamus (reviewed in (Seckl, 1997)). 11β-HSD1 may modulate glucocorticoid actions in the brain, where it has been shown to reactivate 11-dehydrocorticosterone in cultured hippocampal cells, potentiating glucocorticoid-mediated neurotoxicity (Rajan et al., 1996). Furthermore, aged 11βHSD1 knockout mice show significantly lower hippocampal tissue corticosterone levels than wild-type controls and resistance to age-related learning deficits despite elevated plasma corticosterone levels throughout life (Yau et al., 2001b).

11β-HSD1 is also found in omental fat (where it may increase local glucocorticoid activation and promote obesity (Livingstone et al., 2000), ovary and vasculature (reviewed in (Krozowski et al., 1999)).

11β-HSD2 plays a pivotal role in protecting the mineralocorticoid receptor in target tissues, including the kidney, by preventing access of corticosterone and allowing aldosterone to bind. Thus, congenital deficiency in humans (Ferrari et al., 1996) or mice (Kotelevtsev et al., 1999), or inhibition of 11β-HSD by liquorice derivatives causes massively increased MR signalling in these tissues and hence sodium and water retention, potassium loss and severe hypertension ((Ferrari et al., 1996; Holmes et al., 2001; Kotelevtsev et al., 1999), reviewed in (Krozowski et al., 1999)).

In rodents, 11β-HSD2 levels are high in the placenta in mid-gestation then drop towards birth in parallel with expression levels in foetal tissues (Brown et al., 1996). Thus in rodents placental 11β-HSD2 may have a similar role to in humans, where it protects the foetus from high circulating levels of maternal glucocorticoids by inactivating them (reviewed in (Edwards et al., 1993)). Placental 11β-HSD2 dysfunction has been suggested as a potential cause of adult hypertension (reviewed in (Edwards et al., 1993)) and intrauterine growth retardation (reviewed in (Seckl, 1994)) in humans. Bypass of the placental 11β-HSD2 barrier by inhibition with carbenoxolone in pregnant rats leads to changes in GR expression in the amygdala of the offspring and altered behaviour (Welberg et al., 2000). In the adult brain, expression of 11β-HSD2 is confined to regions of the brainstem and the subcomissural organ, which are potential aldosterone targets. However, the high levels of 11β-HSD2 expressed in the foetal brain may serve to protect the developing brain from inappropriate glucocorticoid signalling (reviewed in (Seckl, 1997)). In the vasculature, loss of 11β-HSD2 leads to endothelial dysfunction (Hadoke et al., 2001).

Glucocorticoids are catabolised in the liver by a variety of reduction, oxidation and hydroxylation reactions (Orth, 1998). Most of their metabolites are conjugated and rapidly excreted in the urine (Figure 1.3). In humans (but not rodents), enterohepatic

circulation of glucocorticoids results in excretion of about 15% of secreted cortisol in the faeces (Ganong, 1989). The metabolism of corticosterone is broadly the same as that of cortisol, but 17-keto derivatives are not formed (Ganong, 1989). The balance of glucocorticoid secretion and clearance from the body determines the level of circulating glucocorticoids (Ganong, 1989).

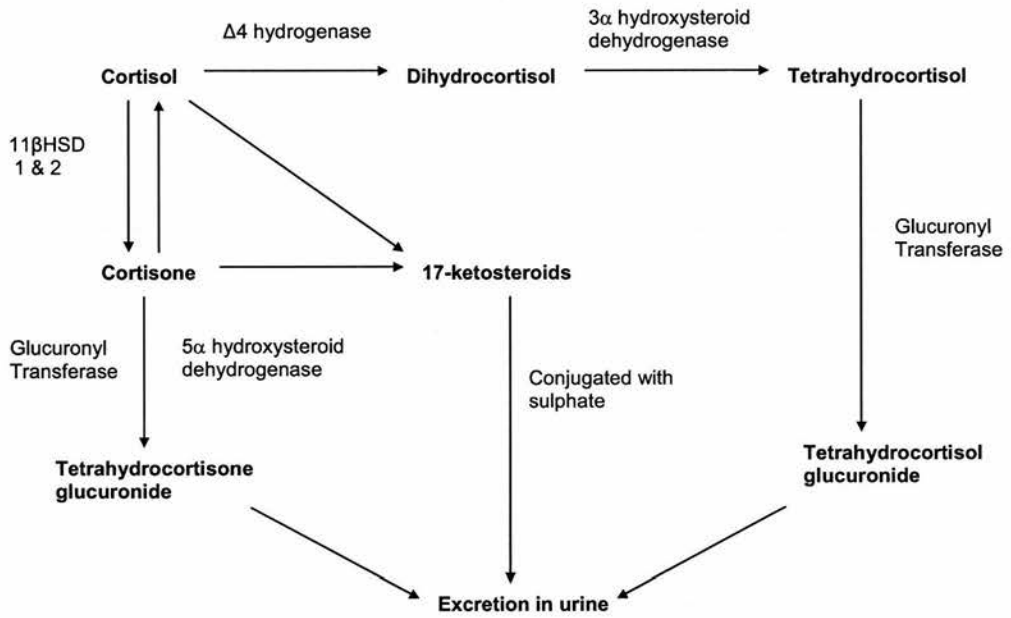


Figure 1.3: outline of the hepatic metabolism of cortisol. Adapted from (Ganong, 1989)

1.4 Mode of action of glucocorticoids

Glucocorticoids act via intracellular corticosteroid receptors, either the type I (mineralocorticoid) receptor (MR) or the type II (glucocorticoid) receptor (GR) (Orth, 1998). The two receptors show specific patterns of expression, both between and within tissues ((Herman et al., 1989a; Reul et al., 1989; Reul and DeKloet, 1985) and reviewed in (McEwen et al., 1986)). Briefly, the GR is ubiquitous and mediates most of the functions of glucocorticoids, although in hippocampus and heart, which lack expression of the enzyme 11β-HSD2 (section 1.3) both receptors bind glucocorticoids. MR has a higher affinity for corticosterone than GR (Feldman et al., 1973). In brain at least, MR has been shown to be an important mediator of glucocorticoid effects, particularly in HPA axis feedback regulation (section 1.9.2.4).

The ubiquitous requirement for GR to mediate the effects of glucocorticoids has been demonstrated in transgenic animals such as $GR^{dim/dim}$ and GR^{AS} mice (section 1.8). Conversely, mineralocorticoid receptor knockout mice show only abnormalities of mineral balance consistent with hypoaldosteronism (Berger et al., 1998) and death of dentate gyrus neurones (Gass et al., 2000). As it is the subject of this thesis, only the GR will be discussed in detail here.

1.5 The glucocorticoid receptor

1.5.1 Structure and function of the glucocorticoid receptor

The glucocorticoid receptor is a member of the nuclear hormone receptor superfamily, which includes all of the classical steroid receptors as well as numerous other receptors for small ligands (retinoic acid and derivatives, thyroid hormone, fatty acids etc.) as well as several receptors for which the ligand has not been identified, the so-called "orphan receptors" (reviewed in (Beato, 1989; Kumar and Thompson, 1999)). The nuclear receptors all contain 4 distinct domains; a variable N-terminal domain, a highly conserved DNA binding domain, a moderately conserved C-terminal or ligand binding domain and a hinge domain (reviewed in

(Beato, 1989; Kumar and Thompson, 1999)). All of these domains are involved in receptor function.

The first event in the classical model of steroid hormone action is binding of the ligand to a cytoplasmic form of the receptor (reviewed in (Beato, 1989; Truss et al., 1992)) which is thought to form a dynamically dissociating and reassociating "foldosome" with various proteins including hsp90, hsp56, hsp70 and p23, maintaining the monomeric GR in a conformation able to bind ligand (reviewed in (Beato et al., 1996; Bertorelli et al., 1998)). The crystal structure of the GR ligand binding domain (LBD) has recently been determined using GR with a point mutation in helix 5 (phenylalanine 602 to serine) bound to dexamethasone and a section of the TIF2 coactivator peptide (Bledsoe et al., 2002). The GR LBD contains 11α-helices and 4 small β-strands that fold into a 3-layer helical domain with helices 1 and 3 on one side of a helical sandwich and helices 7 and 10 on the other side (Bledsoe et al., 2002). This structure is similar to that of the oestrogen, retinoic acid and thyroid hormone receptors (reviewed in (Kumar and Thompson, 1999)). The middle layer of helices (4,5,8 and 9) are absent in the bottom half of the GR LBD, creating the ligand binding pocket which is composed of residues from helices 3, 4, 5, 6, 7, 10 and the AF-2 helix as well as from β-strands 1 and 2 (Bledsoe et al., 2002). Following the AF-2 helix is an extended strand that forms a conserved β-sheet with a β-strand between helices 8 and 9 (Bledsoe et al., 2002).

The conformation of the ligand binding pocket varies between the different nuclear receptors (reviewed in (Kumar and Thompson, 1999)). The structure of the GR steroid pocket seems to be especially distinctive, since unlike the PR, AR and ER the GR pocket has an additional branch extending from the centre which may help to explain the GR selectivity of glucocorticoids (Bledsoe et al., 2002).

The AF2 activation helix of the LBD has been shown to have a ligand-inducible activation function in the oestrogen, thyroid hormone and retinoic acid receptors (Green and Chambon, 1988; Nagpal et al., 1993). On ligand binding the GR AF2 helix is thought to undergo a conformational change from a helix extending away from the core of the LBD to a tight part of the globular LBD (reviewed in (Beato et

al., 1996)). This hypothesis is supported by the fact that in the liganded GR crystal structure discussed above the AF-2 helix adopts the so-called agonist-bound conformation where it packs against helices 3,4 and 10 (Bledsoe et al., 2002). AF-2 also interacts directly with dexamethasone, which may stabilize the AF-2 helix in the active conformation and may serve as a molecular basis for ligand-dependent activation of GR (Bledsoe et al., 2002). The conformational change in AF-2 is thought to lead to dissociation from the GR/hsp complex (reviewed in (Beato et al., 1996)), conformational derepression of the DNA binding domain (Godowski et al., 1987), hyperphosphorylation of the GR protein and translocation into the nucleus (reviewed in (Bertorelli et al., 1998)). In its active conformation AF-2 also participates in one of the 2 charge clamps that GR uses to interact with coactivators (Bledsoe et al., 2002). The second charge clamp is conserved in GR, AR, PR and MR but not in ER or the retinoic acid receptor, so it may account for the differential binding of coactivator motifs by many nuclear receptors (Bledsoe et al., 2002).

It is possible that steroid receptor antagonists exert their effects by preventing AF2 from entering its active conformation; in the oestrogen receptor, the agonist 17ß-oestradiol is buried inside the receptor when bound whereas the antagonist raloxifene enters the hydrophobic core in a different orientation, causing an alteration in the position of the AF2 helix that blocks the LBD binding site for coactivators (Brzozowski et al., 1997).

The GR LBD contains a dimerisation interface consisting of a core hydrophobic interface between residues P625 and I628 in the β-turn of strands 3 and 4 surrounded by an extensive network of hydrogen bonds mediated by the extended strand between helices 1 and 3 and the last residue of helix 5 (Bledsoe et al., 2002). The crystallographically observed GR dimerisation interface is structurally distinct from those of the PPARγ and retinoic acid receptors and allows the GR to form a homodimer with a C2 symmetric packing arrangement (Bledsoe et al., 2002). Mutations of I628 prevent dimerisation and inhibit the ability of GR to activate the MMTV promoter but do not affect GR inhibition of activation by NF-κB (section 1.11.2), suggesting that the monomer and dimer forms of GR may regulate distinct signaling pathways (Bledsoe et al., 2002).

After ligand binding GR translocates into the nucleus, where it binds to DNA as a homodimer (reviewed in (Beato, 1989; Truss et al., 1992)). Recent studies utilizing fusions of GR and green fluorescent protein have elegantly demonstrated that both agonist and antagonist-bound GR translocate into the nucleus (Htun et al., 1996). However, agonist-bound GR appeared to concentrate into a discrete series of nuclear foci, whereas antagonist-bound GR did not (Htun et al., 1996). A short sequence of amino acids towards the C-terminal end of the GR DBD appears to be responsible for nuclear translocation: another similar sequence lies in the carboxy-terminal half of the DBD that overlaps the LBD (Picard and Yamamoto, 1987). Nuclear translocation of the GR requires an intact cytoskeleton (Galigniana et al., 1998) and thus presumably involves microtubule/microfilament systems.

GR binds to DNA via the highly-conserved DNA binding domain (DBD), which is the most conserved region amongst the nuclear receptors. The DNA sequence recognized by the DBD is a 15-mer partially palindromic consensus sequence, the glucocorticoid response element or GRE (5'-GGTACAnnnTGTTCT-3'). The DBD also contacts an additional turn of the DNA double helix on each side of the GRE (reviewed in (Beato, 1989)). Both positive and negative (5'-ATYACNnnnTGATCW-3') GREs exist, with the positive GRE also being also being able to mediate induction by progesterone receptor (PR), AR and MR binding ((Strahle et al., 1988), reviewed in (Beato, 1989)). The structures of the GR DBD in solution (Hard et al., 1990) and bound to DNA (Luisi et al., 1991) have been determined and are very similar. The main feature of the secondary structure of the GR DBD is 3 helical regions (Figure 1.4).

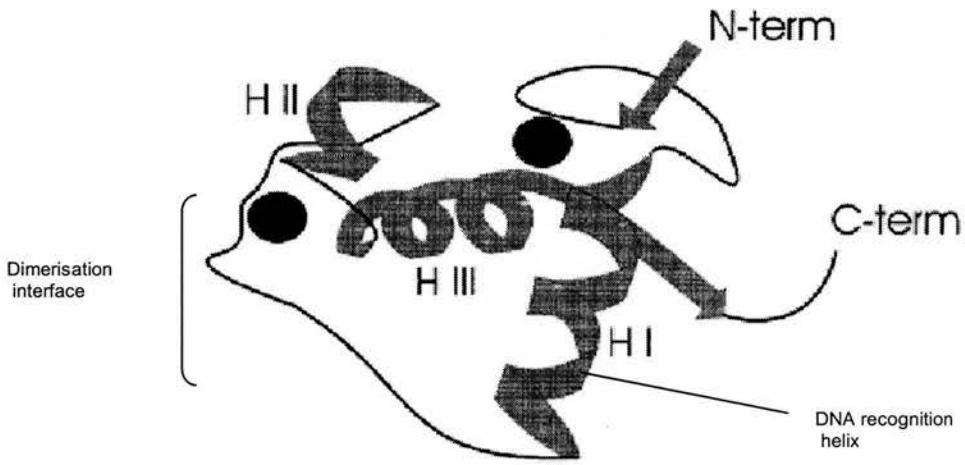


Figure 1.4: structure of the glucocorticoid receptor DNA binding domain.

The two Zn ions are represented by filled circles. HI-HIII are the amino acid helices. Adapted from (Kumar and Thompson, 1999).

Helices I and II are α-helices (Hard et al., 1990) and lie perpendicular to each other at the base of a hydrophobic core (Luisi et al., 1991), while helix III is somewhat distorted (Hard et al., 1990). The DBD of each GR monomer contains 2 "zinc finger" motifs (consisting of 4 highly conserved cysteine residues coordinating binding of a Zn atom) and can be considered as two structurally and functionally distinct subdomains, each nucleated by one of the zinc coordination centres followed by an amphipathic protein helix ((Luisi et al., 1991), reviewed in (Kumar and Thompson, 1999)). The first subdomain mediates site-specific recognition of DNA, especially via 3-4 amino acid residues in helix I termed the P box (Luisi et al., 1991). During DNA binding helix I lies in the major groove of the DNA (Luisi et al., 1991). The second subdomain contains a sequence of amino acids termed the D box which is important for protein-protein interactions in the dimeric DBD:GRE complex (Luisi et al., 1991). A single lysine residue in the DBD may act as an "allosteric lock", interpreting GRE signals by serving as a determinant of GR conformation and function and thus preventing transcriptional activation by GR at sites other than positive GREs (Starr et al., 1996). However, the structure of the DBD may be somewhat flexible and may undergo a degree of rearrangement during DNA binding (reviewed in (Kumar and Thompson, 1999)).

The N-terminal domain of GR contains a powerful transactivation domain termed activation function 1 (AF1/tau1/enh2) that plays an important role in gene regulation (Giguere et al., 1986; Hollenberg et al., 1987). This region is largely unstructured in aqueous solution but forms α-helices in the presence of trifluoroethanol (Dahlman-Wright et al., 1995). Mutational studies show that the ability of this domain to activate a reporter gene *in vivo* correlates with ability to form α-helices *in vitro* and that hydrophobic residues in these α-helices are important in transactivation (Dahlman-Wright et al., 1995; McEwan et al., 1993). However, the mechanism by which AF1 might adopt a α-helical conformation *in vivo* is unclear. It also unclear how AF1 interacts with other proteins to induce transcription. However, AF1 has been shown to physically interact with the transcriptional machinery, specifically

with the Ada2 and TATA-binding protein subunits (Ford et al., 1997; Henriksson et al., 1997). It may do so by an induced fit mechanism, leading to the formation of a stable complexed structure (Tjian and Maniatis, 1994). Interactions with coactivators are also likely to be important (reviewed in (Kumar and Thompson, 1999)). Transcriptional activation and repression by GR is discussed in detail in section 1.11.

In addition, distinct membrane-associated GRs may also exist (Harrison et al., 1979; Hua and Chen, 1989; Orchinik et al., 1991; Sadler et al., 1985), signalling via G proteins (Iwasaki et al., 1997) and possibly mediating some of the rapid non-genomic effects of glucocorticoids that have been observed (Evans et al., 1998; Iwasaki et al., 1997). As they are unlikely to be related to the classical GR, they will not be discussed further here.

1.5.2 GR isoforms in human cells

The isolation of 2 human GR cDNA clones predicted the existence of 2 receptors with different carboxyl termini (Hollenberg et al., 1985). The first 727 amino acids of the 2 isoforms are the same, but exons 9α and 9β undergo alternate splicing with 9α (in GRα mRNA) coding for 50 amino acids while 9β (in GRβ mRNA) codes for 15 nonhomologous amino acids (Encio and Detera-Wadleigh, 1991). Exons 9α and 9β may form 1 large exon encoding the 3' end of the LBD and containing approximately 4kb of 3' untranslated sequence (Oakley et al., 1996). The structure of the GR gene is discussed in section 1.6.

GRα is the predominantly expressed isoform, although 2 variant GRα mRNA transcripts of 7 and 5.5kb have been reported. Both are widely distributed but the 7kb variant is more abundant and the 5.5kb variant is particularly scarce in brain (Oakley et al., 1996). GRβ mRNA is widely expressed in human tissues although it is approximately 200-500 times less abundant than GRα mRNA (Oakley et al., 1996). Homologous genomic sequences for exon 9β have been found in the rat GR cDNA sequence and RT-PCR products from rat liver and a mouse lymphoma cell line (Encio and Detera-Wadleigh, 1991). However, the presumed mouse exon 9β contains an open reading frame of 59 amino acids whereas human exon 9β encodes only 15 (Otto et al., 1997) and RT-PCR failed to detect GRβ mRNA in mice (Otto et al.,

1997). Furthermore, the rat and mouse sequences homologous to exon 9β are not preceded by a splice site (Otto et al., 1997). Thus it is not yet known if the GRβ mRNA is translated into protein *in vivo* and, if so, what the relative levels of GRα and GRβ are in the cell.

Studies with transfected GRβ have shown that it cannot bind the GR ligands dexamethasone or RU38486 (Oakley et al., 1996). Its natural ligand, if any, is unknown. In contrast to ligand-free GRα (section 1.6) it resides primarily in the nucleus (Oakley et al., 1996) although it can interact with hsp90, albeit with a lower affinity than GRα (Oakley et al., 1999). GRβ has been reported to bind DNA with a greater affinity than GRα in the absence of ligand, although in contrast to GRα the presence of glucocorticoids does not enhance this binding affinity (Oakley et al., 1999). GRβ inhibits activation of the mouse mammary tumour virus (MMTV) promoter (section 1.6) by an unknown mechanism (Oakley et al., 1996). GRβ can form heterodimers with GRα (Oakley et al., 1999) and thus may interfere with GRα activity. Alternatively, GRβ may compete with GRα for GREs or for cofactor binding (Oakley et al., 1996) (section 1.6). Whatever the precise mechanism, the dominant negative activity of GRβ is dependent on the terminal 15 amino acid residues (Oakley et al., 1999). The effects of GRβ on GR-mediated transcriptional activation are yet to be demonstrated *in vivo* and the existence of GRß has not been demonstrated in other species than humans, so its physiological significance is uncertain.

Recent work has demonstrated the presence of an additional, slightly smaller, 91 kDa GR protein not recognized by antibodies specific for GRβ in human cells (Yudt and Cidlowski, 2001). When a single human GR cDNA is expressed in COS-1 cells or by *in vitro* transcription two forms of GR are observed, similar to those seen in cells that contain endogenous GR (Yudt and Cidlowski, 2001). These data suggest that two forms of GRα (GR-A and GR-B) are produced by alternative translation of the same gene. Mutagenesis of an internal ATG codon corresponding to methionine 27 (M27) to ACG in human, rat, and mouse GR cDNA resulted in production of GR-A, while mutagenesis of the initial ATG codon to ACG resulted in production of GR-B (Yudt and Cidlowski, 2001). A leaky ribosomal scanning mechanism may be responsible

for generating these two isoforms (Yudt and Cidlowski, 2001). The two isoforms exhibit similar subcellular localization and nuclear translocation after ligand activation but functional analyses show the shorter GR-B to be nearly twice as effective as the longer GR-A in gene transactivation, but not in transrepression (Yudt and Cidlowski, 2001). The physiological significance of this is as yet unknown.

1.6 The glucocorticoid receptor gene

The GR gene is large and complex (Figure 1.5). Originally cloned in 1985, the human glucocorticoid receptor cDNA spans approximately 100kb and contains 9 exons; a non-coding exon (now designated 1C) and 8 protein-coding exons (2-9) (Hollenberg et al., 1985). The N-terminal end of the protein is encoded by exon 2, the zinc fingers are encoded by exons 3 and 4, exon 5 encodes residues involved in transactivation and exons 6-8 encode the ligand-binding domain (Encio and Detera-Wadleigh, 1991). Recent evidence suggests that at least 2 other alternate exons 1 exist within GR mRNA from human muscle (Reynolds R, personal communication) and at least 5 variant exons 1 are present in the human MOLT-4 T cell leukaemia cell line, one of which lies at least 31kb upstream of the translation start (Breslin et al., 2001). Primer extension, ribonuclease protection (Zong et al., 1990) and S1 nuclease analysis (Encio and Detera-Wadleigh, 1991) of the human GR mRNA reveals at least 4 transcription start sites for exon 1C. The 5' flanking region of this exon (to –860bp, with ATG designated as +1) contains no obvious TATA or CAAT boxes but contains multiple GC boxes which are bound by SP1 and related factors (Encio and Detera-Wadleigh, 1991; Zong et al., 1990). Additionally, transcription start sites in the human GR gene corresponding to the rat exons 1_6, 1_{10} and 1_{11} have recently been identified (Suzuki et al., 2002). Transfection experiments have shown differential activity of promoters 1B and 1C in various cell lines (Nunez and Vedeckis, 2002), while DNase I footprint and gel mobility shift analysis reveals that AP2 may be an important activator of the gene (Nobukuni et al., 1995). Also, the human GR gene promoter contains four binding sites for the transcription factor YinYang1 (Breslin and Vedeckis, 1998) and several SP1 binding sites (Breslin and Vedeckis, 1998; Nunez and Vedeckis, 2002). As well as the 5' alternate exons described above, 3' alternate exons of the GR gene may also exist in humans (section 1.5.2).

The mouse GR gene contains at least 5 alternate exons 1 (Chen et al., 1999b; Strahle et al., 1992), 4 of which lie within a CpG island (1B-1E) while 1 (1A) lies approximately 32kb upstream of the translation start site (Strahle et al., 1992). At least 3 of the alternate exons (1A-1C) show considerable 5' heterogeneity (Strahle et al., 1992). Two of the alternate exons are ubiquitously expressed (1B and 1C) while 1A may be restricted to T lymphocytes, since it is found in T-lymphoma cell lines but not in lines derived from a range of other tissues (Strahle et al., 1992). However, recent evidence suggests that exon 1A may be more widely distributed (Chen et al., 1999b) and one report suggests it may be involved in targeting GR to the cell membrane (Gametchu et al., 1999). Exons 1D and 1E are widely expressed (Chen et al., 1999b) and DNase hypersensitive sites are found upstream of exons 1B and 1C in liver and fibroblast cells, consistent with ubiquitous activity of their putative associated promoters (Strahle et al., 1992).

The rat GR gene has at least 12 alternate exons 1 (Gearing et al., 1993; McCormick et al., 2000): 5 of these correspond to those identified in the mouse GR gene and two correspond to the human exons 1B and 1C (Figure 1.5). 10 of these alternate exons 1 lie in a 3kb CpG island and 3 are likely to lie over 15kb 5' of the translation start as they are not present in the rat genomic clone λ208, which contains exon 2 and approximately 15kb of its 5' flanking sequence (McCormick et al., 2000). In the rat, the alternate exons 1 of the GR gene vary in their tissue-specific relative abundance. In the hippocampus, approximately 60% of GR mRNA contains exon 1_{10} and approximately 20% contains exon 1_6; the remainder of the GR mRNA pool contains 3 other exon 1 variants ($\approx$ 8% exon 1_5, $\approx$ 8% exon 1_7 and $\approx$ 10% exon 1_{11}) (McCormick et al., 2000). In liver, 3 exons 1 are present in most GR mRNA; $\approx$ 10% contains exon 1_6, $\approx$ 75% contains exon 1_{10} and $\approx$ 2% contains exon 1_{11} (McCormick et al., 2000). Most of the GR mRNA in thymus contains exon 1_{10} ($\approx$ 55%) with most of the remainder containing either exon 1_1 ($\approx$ 25%) or exon 1_6 ($\approx$ 20%) (McCormick et al., 2000). Interestingly, expression of some variants appears restricted to certain tissues: exons 1_5 and 1_7 were found in hippocampus but not liver and thymus, while exon 1_1 was found in thymus but not hippocampus and liver (McCormick et al., 2000).

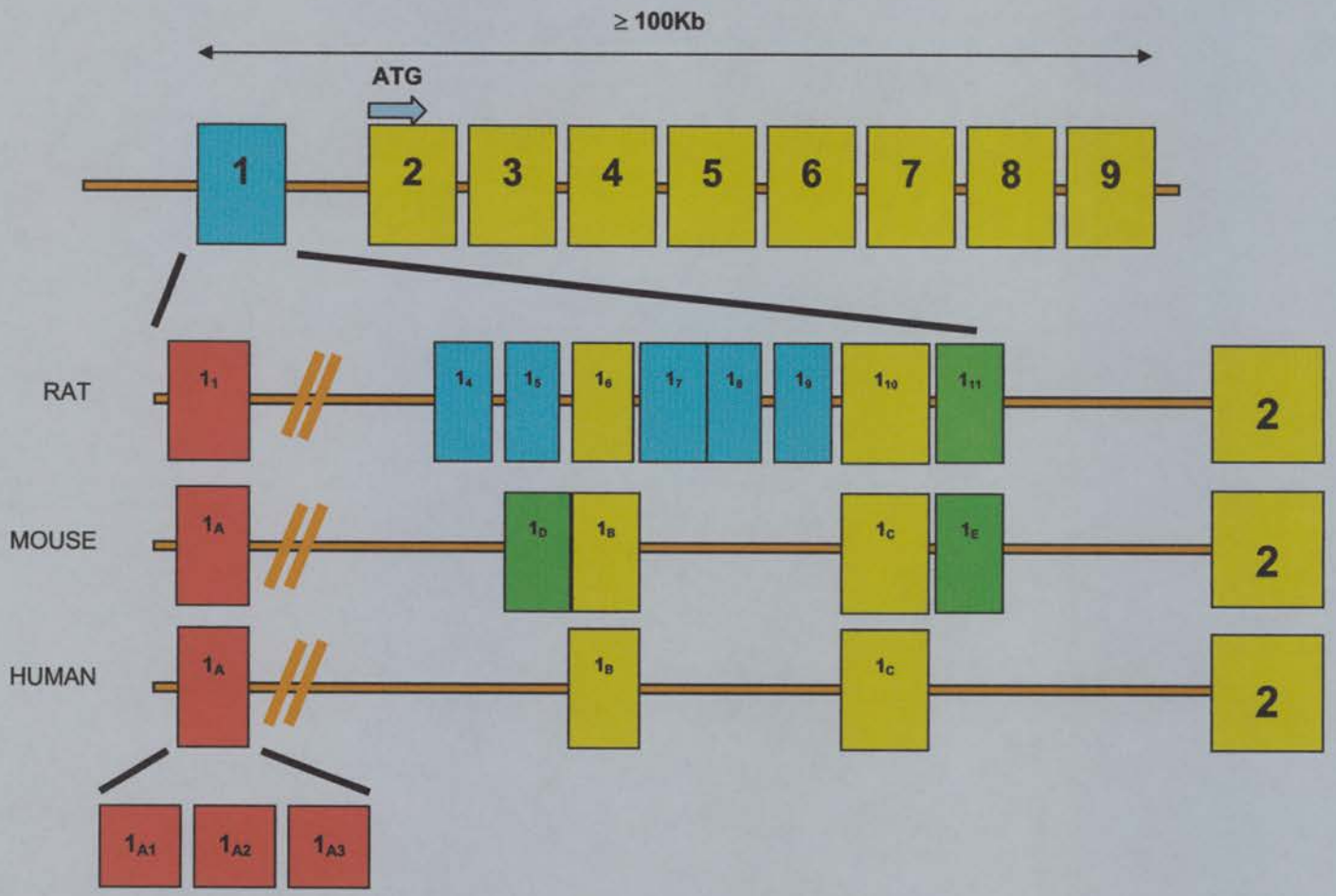


Figure 1.5: structure of the glucocorticoid receptor gene in rat, mouse and human.

1.7 Control of GR gene expression

There is much evidence that the level of GR in the cell is a major determinant of the level of glucocorticoid signaling in tissues and that the correct level of functional GR in tissues is critical for its correct physiological function. Overexpression of GR leads to increased glucocorticoid sensitivity, while reduced amounts of functional GR in the cell leads to glucocorticoid resistance. For example, *in vitro* experiments show that the level of GR expression in a cell correlates with its glucocorticoid sensitivity (Vanderbilt et al., 1987). Also, a heterozygous missense mutation in the GR caused glucocorticoid resistance due to inhibited GR-mediated transcriptional activation and nuclear translocation of GR, resulting in pituitary Cushing's Disease (Karl et al., 1996). Other human GR mutations are associated with malfunctioning GR in asthma patients and a frameshift mutation in GR is associated with glucocorticoid resistance in Nelson's Syndrome (reviewed in (Tenbaum and Baniahmad, 1997)).

Further evidence for the importance of the level of GR in the cell comes from transgenic animals, which are further discussed in section 1.8. For example, GR^{AS} mice have a 50-70% reduction in GR levels compared to wild-type controls and show impaired sensitivity of their HPA axis to glucocorticoid negative feedback (Pepin et al., 1992b). The lungs, liver and adrenal glands of $GR^{hypo/hypo}$ mice, which lack normal GR protein, fail to respond normally to glucocorticoids during development and these mice show decreased sensitivity of their HPA axis to glucocorticoid negative feedback (Cole et al., 1995a). Furthermore, reduced GR levels in the thymocytes of $GR^{hypo/hypo}$ and $GR^{AS(Thy)}$ mice causes them to be resistant to glucocorticoid-mediated apoptosis (Cole et al., 1995a; King et al., 1995). These data support the hypothesis that reduced intracellular GR leads to glucocorticoid resistance. Furthermore, an increase in GR sensitizes tissues to the effects of glucocorticoids. YGR mice (section 1.8.6) variably overexpress GR in their tissues (e.g. by 170% in the hippocampus) due to the insertion of a YAC transgene, resulting in increased sensitivity of their HPA axis to glucocorticoid negative feedback and increased sensitivity of their thymocytes to glucocorticoid-mediated

apoptosis (Reichardt et al., 2000b). These data support the hypothesis that increased intracellular GR leads to increased glucocorticoid sensitivity.

1.7.1 Transcriptional regulation of the GR gene

Much data is available on the dynamic regulation and perinatal programming of GR levels in various tissues (sections 1.7.2 and 1.7.3), in which GR clearly has an important role. Although the mechanisms by which GR regulates transcription have been extensively studied (section 1.11), surprisingly little is known about the molecular mechanisms regulating expression of the GR gene.

Studies of GR expression in rat liver (Dong et al., 1988) as well as rat pancreatic tumour (Rosewicz et al., 1988), rat hepatoma (Dong et al., 1988) and human lymphoma (Rosewicz et al., 1988) cells suggest that the control of GR levels is probably at the level of transcription. However, the observation that dexamethasone reduces the half-life of GR protein in rat hepatoma cells (Dong et al., 1988) and that acute stress alters the availability of GR mRNA without demonstrably affecting transcription (Paskitti et al., 2000) suggests that some post-transcriptional regulation may occur.

The expression of multiple variant GR exons 1 in rat, mouse and human, the report that one promoter in the human GR gene (promoter 1A3) is activated by glucocorticoids (Breslin et al., 2001) and the differential activity of promoters 1B and 1C in different cell lines (Nunez and Vedeckis, 2002) suggest that tissue-specific control of GR levels may involve alternate promoter usage (section 1.10.2). Indeed, increased activity of the human promoter 1A has been implicated in T cell apoptosis and T cell maturation (Chen et al., 1999b; Chen et al., 1999a) and glucocorticoid autoregulation of promoter 1A activity has been reported (Breslin et al., 2001).

In the human GR gene, a region between 250bp and 750bp 5' of the transcription start of exon 1C (section 1.6) is implicated in autoregulation and is bound by an unidentified protein (Leclerc et al., 1991). The transcription factors AP2 (Nobukuni et al., 1995), YinYang1 (Breslin and Vedeckis, 1998) and SP1 (Breslin and Vedeckis, 1998; Encio and Detera-Wadleigh, 1991; Nunez and Vedeckis, 2002;

Zong et al., 1990) may be important in regulation of human GR expression. Also, a mechanism for autoregulation of the human GR gene has been proposed in which ligand-bound monomeric GR interacts with the jun component of AP1, reducing induction of GR expression by reducing AP1 binding to the GR promoter (Vig et al., 1994).

1.7.2 Dynamic regulation of GR expression

1.7.2.1 Glucocorticoid autoregulation of GR

Glucocorticoids are reported to regulate GR expression in most tissues in which autoregulation has been investigated. The regulation of GR expression by glucocorticoids has been studied most extensively in the hippocampus, since it contains high levels of GR (section 1.9.2) and is an important site of glucocorticoid feedback on the HPA axis (section 1.9.2.4). Interpretation of the various studies requires a degree of care, however, since a wide range of experimental methodologies are employed.

Adrenalectomy (i.e. a reduction in circulating glucocorticoids) of up to 8 days duration increased hippocampal GR mRNA in rats (Herman et al., 1989a; Herman and Spencer, 1998; Holmes et al., 1995b; Reul et al., 1989; Sheppard et al., 1990). Adrenalectomy also increased hippocampal glucocorticoid binding capacity in rats (Lowy, 1989; Spencer et al., 1991).

Initially, increased hippocampal total GR mRNA (Kalinyak et al., 1987) and glucocorticoid binding capacity (Reul et al., 1987a) were reported 2 weeks after adrenalectomy, suggesting that the effects of adrenalectomy were permanent. However, this was disputed by later studies. In one, hippocampal GR mRNA levels 6h and 2d after adrenalectomy were elevated, yet those 14d after adrenalectomy were not different from control animals (Holmes et al., 1995b). Another study demonstrated a transient rise in hippocampal GR mRNA that was abolished by 3d post-adrenalectomy (Reul et al., 1989). These data suggest that the level of GR in the hippocampus may initially rise after adrenalectomy then return slowly to control levels.

The administration of exogenous glucocorticoids reduces hippocampal GR levels in both adrenalectomised and adrenally-intact animals. Corticosterone replacement given to adrenalectomised rats orally over 5 days (Sheppard et al., 1990) or by subcutaneous pellets over 4 (O'Donnell et al., 1995), 6 (Spencer et al., 1991), or 7 days (Reul et al., 1987a) at levels similar to physiological either abolished (O'Donnell et al., 1995; Sheppard et al., 1990; Spencer et al., 1991) or significantly reduced (Reul et al., 1987a) the adrenalectomy-induced rise in hippocampal GR. Similar doses of corticosterone reduced the GR levels in adrenally intact animals (Sapolsky et al., 1984b; Sapolsky and McEwen, 1985; Spencer et al., 1991). Corticosterone replacement at higher doses administered orally, by injection or by subcutaneous pellet either reduced (Peiffer et al., 1991a) or had no effect on (Sheppard et al., 1990) hippocampal GR mRNA and reduced hippocampal glucocorticoid binding sites (O'Donnell et al., 1995; Patacchioli et al., 1998; Sapolsky et al., 1984b; Sapolsky and McEwen, 1985; Tornello et al., 1982). Higher replacement doses also reduced hippocampal GR protein levels (O'Donnell et al., 1995). Thus it appears that the effects of corticosterone are dose-dependent. Indeed, one of the above studies clearly showed a dose-dependent effect of corticosterone replacement in adrenalectomised animals on hippocampal GR binding sites, with higher replacement doses resulting in reduced GR binding sites compared to controls (Spencer et al., 1991). Corticosterone also downregulates GR mRNA in cultured hippocampal neurones (Erdeljan et al., 2001) and reduces glucocorticoid binding capacity in cultured rat neocortical and hippocampal cells (Vedder et al., 1993).

Similarly, dexamethasone administration abolishes the adrenalectomy-induced rise in GR mRNA (Herman et al., 1989a; Holmes et al., 1995b; Peiffer et al., 1991a; Reul et al., 1989), variably reduces hippocampal GR mRNA in adrenally intact animals (Sheppard et al., 1990) and downregulates GR mRNA in cultured hippocampal neurones (Erdeljan et al., 2001). Like corticosterone, the effects of dexamethasone seem to vary with dose, since the magnitude of the reduction in hippocampal GR mRNA in adrenalectomised animals increased as the amount of dexamethasone administered increased (Reul et al., 1987a).

Although several studies report that adrenalectomy increases GR mRNA in CA1/2 and dentate gyrus with corticosterone replacement abolishing this effect (Chao et al., 1998a; Herman et al., 1989a; Herman and Spencer, 1998; Holmes et al., 1995b), only two of these four studies report that glucocorticoid manipulation affects GR mRNA levels in CA3 (Chao et al., 1998a; Holmes et al., 1995b). Thus there may be a region-specific difference in the magnitude of the effect of glucocorticoids on hippocampal GR levels. This may reflect the distribution of GR in the hippocampus, since glucocorticoid manipulation appears to have less effect in areas where is there is less GR (section 1.9.2).

Several studies have investigated whether hippocampal GR expression is regulated by glucocorticoids via MR or GR. Both dexamethasone and corticosterone will bind to either receptor, although dexamethasone has a higher affinity for GR (Arriza et al., 1988; Spencer et al., 1990) and corticosterone has a higher affinity for MR (Spencer et al., 1990). Since the hippocampus lacks 11ß-HSD2 (Robson et al., 1998) the MR there are open to corticosterone binding (section 1.3). Thus the effects of dexamethasone described above are likely to be occurring via GR, while those of corticosterone could theoretically be modulated via either receptor. Indeed, administration of the specific MR ligand aldosterone or the specific GR ligand RU28362 both attenuate the adrenalectomy-induced increase in hippocampal GR mRNA levels in CA1, while in CA3 only aldosterone has this effect (Chao et al., 1998a). Also, administration of the MR antagonist spironolactone to adrenally-intact animals increased GR mRNA and protein in CA1 and DG to levels similar to those in adrenalectomised animals, while corticosterone administration to the same animals had no effect on GR mRNA levels (Herman and Spencer, 1998). Taken together with the data from adrenalectomy/replacement experiments, these data suggest that *in vivo* autoregulation of hippocampal GR by corticosterone is occurring via both GR and MR. Due to their relative affinities for corticosterone (see above) and different abundances in the hippocampus (section 1.9.2.4), it is likely that at basal corticosterone levels MR signalling predominates, while at higher glucocorticoid levels GR signalling becomes more important (reviewed in (Seckl and Olsson, 1995)).

Glucocorticoids also regulate GR levels in other tissues. In the liver, adrenalectomy increased GR mRNA expression in rats (Kalinyak et al., 1987; Reul et al., 1989) and caused a 2-2.5 x increase in hepatic glucocorticoid binding capacity in mice (Svec et al., 1989). However, the effect of adrenalectomy may be less pronounced than in brain since in rats hepatic GR was increased by only 15% 2 weeks after adrenalectomy compared to a 40% increase in brain (Kalinyak et al., 1987). Hepatic GR in mice may be more sensitive to glucocorticoid regulation since 24h adrenalectomy caused a 2-2.5 x increase in hepatic glucocorticoid binding capacity (Svec et al., 1989). However, this may have reflected loss of endogenous ligand from the receptors, making them available for binding. Corticosterone replacement in adrenalectomised rats reduced hepatic GR binding capacity compared to adrenalectomised controls (Alexandrova et al., 1989) and intraperitoneal injection of 1mg of corticosterone in adrenalectomised mice downregulated hepatic GR number by 25% compared to adrenalectomised controls (Svec, 1988). Dexamethasone treatment of adrenal-intact rats also reduces hepatic GR mRNA levels by approximately 40% (Kalinyak et al., 1987). Furthermore, stress reduces GR levels in the liver of adrenally intact rats, probably due to increased circulating corticosterone levels (Alexandrova and Farkas, 1992). Overall, these data suggest that glucocorticoid regulation of GR levels in the liver is broadly similar to that in the hippocampus.

In the immune system, however, there is some controversy as to the precise effects of glucocorticoids on GR levels. One study reported that 6d adrenalectomy had no effect on thymic GR levels compared to 24h adrenalectomy (Spencer et al., 1991) and another reported that 16h adrenalectomy had no effect on thymic GR levels compared to intact animals (Miller et al., 1990). This might be due to paracrine production of glucocorticoids in the thymus ((Lechner et al., 2000), section 1.9.5.2) which could maintain their local concentration. This hypothesis is supported by the fact that adrenalectomy increased GR mRNA in the spleen compared to adrenal-intact animals (Miller et al., 1990). However, another study reported a 50% increase in thymic GR mRNA after 6d adrenalectomy (Peiffer et al., 1994). One explanation for this discrepancy could be that there is some post-transcriptional control of GR

levels in the thymus, although evidence from other tissues suggests that this is unlikely (section 1.7.1). Alternatively, as postulated in the hippocampus (see above), the effects of adrenalectomy may vary in the thymus over time.

The effects of glucocorticoid administration in the thymus are also controversial. In one study, corticosterone replacement caused a reduction in thymic GR binding capacity and a dose-dependent reduction of GR binding capacity in the spleen (Spencer et al., 1991). However, a stress-induced rise in corticosterone sufficient to reduce GR levels in the hippocampus by 25% had no effect in the thymus (Miller et al., 1990). Also, glucocorticoids have been shown to increase total GR mRNA in the human CEM-C7 T cell line (Antakly et al., 1989; Ashraf et al., 1991; Denton et al., 1993; Eisen et al., 1988). One explanation for this could be that glucocorticoids upregulate GR in these cells as they undergo apoptosis ((Ramdas et al., 1999; Tonko et al., 2001), section 1.9.5.2).

1.7.2.2 Regulation of GR by other hormones and neurotransmitters

Perhaps the most important neurotransmitter that dynamically influences GR expression is 5-HT: it may also have an important role in perinatal programming of GR expression (section 1.7.3). 5-HT upregulated GR mRNA (Erdeljan et al., 2001; Hery et al., 2000; Mitchell et al., 1990b; Mitchell et al., 1992) but not MR mRNA (Erdeljan et al., 2001; Mitchell et al., 1990b) in cultures of foetal rat hippocampal neurones. There is also evidence that 5-HT regulates GR (Seckl et al., 1990) and MR (Yau et al., 1997b) mRNA in hippocampal neurons *in vivo*.

The interplay between hippocampal glucocorticoid and 5-HT signalling and receptor regulation *in vivo* appears to be extremely complex. Reduced hippocampal 5-HT levels due to lesions of the serotonergic innervation of the hippocampus downregulate hippocampal GR but not MR mRNA (Seckl et al., 1990). However, treatment with the selective 5-HT neurotoxin 3,4-methylenedioxymetamphetamine (which causes both corticosterone and 5-HT release) decreases GR and upregulates MR mRNA (Yau et al., 1997b). Also, the effects of these manipulations are greater in the dentate gyrus than other hippocampal subfields (Seckl et al., 1990; Yau et al.,

1997b). The effects of 3,4-methylenedioxymetamphetamine on GR expression seem to require both serotonin and corticosterone release throughout the hippocampus, while its effects on MR expression seem to require corticosterone release only in CA1-4 (Yau et al., 1997b). Thus it appears that there is a considerable degree of crosstalk between the 5-HT and GR receptor systems in the hippocampus.

The mechanism by which 5-HT regulates GR and MR expression is not fully understood. However, there is evidence that it may act via the $5HT_2$ and/or $5HT_{1C}$ receptors to cause an increase in intracellular cyclic AMP levels (Mitchell et al., 1990b; Mitchell et al., 1992).

Several other neurotransmitters downregulate hippocampal GR, e.g. those acting via NMDA receptors (such as glutamate, aspartate and glycine), gamma-aminobutyric acid and adrenaline (Tritos et al., 1999). Given that stress modulates the expression of many receptor proteins in the hippocampus such as growth hormone receptor (Fujikawa et al., 2000), the gamma-amino butyric acid A receptor (Cullinan and Wolfe, 2000) and MR (Fujikawa et al., 2000), these neurotransmitters are likely to mediate (along with the effects of 5-HT described above) many of the effects of stress on hippocampal GR expression.

GR expression may be regulated by sex hormones, particularly oestrogen. Although gonadally-intact male and female rats showed no differences in hippocampal GR and MR number (Turner, 1992), treatment with oestrogen resulted in a reduction of hippocampal GR number in ovariectomised and adrenalectomised female rats (Turner, 1992). However, another study reported no change in hippocampal GR with oestrogen treatment and a downregulation of MR in the pituitary (Ferrini et al., 1990). Nevertheless, these data suggest that the effect of oestrogen on GR in the normally-cycling female rat is relatively small and the mechanism by which oestrogen might affect GR mRNA expression is unknown. Conversely, thymic GR mRNA levels varied significantly during the oestrus cycle of rats, with the highest levels in oestrus and the lowest in proestrus. This suggests that the effects of oestrogen on GR level are tissue-specific. GR levels may also be regulated by androgens. In the hippocampus, testosterone administration to castrated rats

downregulated GR mRNA by approximately 30% in CA1 compared to intact animals (Kerr et al., 1996). Conversely, in the rest of the hippocampus (which, unlike CA1, has little or no expression of the androgen receptor) testosterone had no effect (Kerr et al., 1996).

1.7.2.3 Pharmacological regulation of GR

Tricyclic antidepressants can increase GR mRNA levels in cultured primary hippocampal (Pepin et al., 1989), mouse fibroblast and mouse neuroblastoma cells (Barden, 1996). They have similar effects *in vivo*; in rats imipramine and desipramine increased hypothalamic GR mRNA levels (Peiffer et al., 1991b) and amytryptiline and desipramine increased hippocampal MR and GR mRNA (Seckl and Fink, 1992). Amytryptiline and desipramine also increase hippocampal glucocorticoid binding sites (Okugawa et al., 1999).

The effects of antidepressants on GR and MR expression may be timecourse-specific, since amytryptiline and desipramine show no effect on GR mRNA after 2d treatment but do after 14d (Seckl and Fink, 1992). The effects of antidepressants may also be drug-specific. Chronic lithium chloride injection increased hippocampal GR but not MR mRNA (Semba et al., 2000), citalopram did not affect hippocampal GR mRNA at all (Seckl and Fink, 1992) and 9d treatment with the selective serotonin reuptake inhibitors fluoxetine and venlafaxine had an oppsite effect to the tricyclic antidepressants, downregulating MR mRNA throughout the hippocampus and GR mRNA specifically in CA3 (Yau et al., 2001a). Also, the effects seem to be receptor-specific since amytryptiline administration affected MR expression more quickly than GR expression (Seckl and Fink, 1992).

5HT regulates GR in hippocampal neurones (section 1.7.2.2), suggesting that these drugs may exert their effects by altering hippocampal 5HT levels. However, amytryptiline and desipramine have acute effects on GR expression which may be due to a direct effect on the cell (Barden, 1996; Okugawa et al., 1999), as does citalopram (Hery et al., 2000). In particular, desipramine may facilitate GR translocation and function (Pariante et al., 1997).

1.7.3 Perinatal programming of GR expression

Over the last decade the "foetal origins hypothesis" that early life events can "program" the development of tissues and organs, thus influencing adult physiology and disease (Barker, 1990) has gained wide acceptance. Based on the observations that low birth weight in humans correlates with adult hypertension (Barker et al., 1990), glucose intolerance (Hales et al., 1991), non-insulin dependent diabetes mellitus (McCance et al., 1994) and ischaemic heart disease (Barker, 1991; Valdez et al., 1994), this hypothesis was linked to glucocorticoid exposure by the observations that administration of glucocorticoids to pregnant humans and rats throughout pregnancy leads to reduced birthweight of offspring (Reinisch et al., 1978) and that foetal cortisol levels are elevated in intrauterine growth disorder (Goland et al., 1993). Dexamethasone administration in the third trimester of pregnancy causes reduced birthweight (Levitt et al., 1996; Nyirenda et al., 1998) as well as high blood pressure (Levitt et al., 1996) and fasting and post-glucose hyperglycaemia (Nyirenda et al., 1998) in adult offspring. The effects of dexamethasone on carbohydrate metabolism are associated with increased PEPCK and GR mRNA in the periportal region of the liver, a major site of hepatic gluconeogenesis (Nyirenda et al., 1998). Taken together, these data suggest that increased glucocorticoid exposure *in utero* may be responsible for programming in humans and rats. The precise mechanism by which the programming of hepatic GR levels by glucocorticoids occurs is unknown. Recent investigations in our laboratory suggest that changes in alternate promoter usage may be involved, since prenatal glucocorticoid exposure decreases the proportion of hepatic GR mRNA containing exon 1_{10} of the GR gene (section 1.6), suggesting an increase in usage of a minor exon 1 variant (McCormick et al., 2000).

In contrast to the effect in the liver, foetal overexposure to glucocorticoids due to *in utero* dexamethasone exposure in the third trimester of pregnancy permanently decreased GR and MR mRNA in the hippocampus (Levitt et al., 1996) while maternal restraint stress reduced hippocampal GR and MR levels in the offspring (Henry et al., 1994). The programming effect appears to be region and receptor-specific since the reduction in GR mRNA due to dexamethasone exposure was confined to CA1 and dentate gyrus while that in MR mRNA was confined to CA1

and CA2 (Levitt et al., 1996). Interestingly, animals exposed to dexamethasone *in utero* throughout pregnancy showed no change in their hippocampal GR and MR levels (Welberg et al., 2001), suggesting that the programming effect only occurs if the hormonal environment of the foetus is different during the last trimester to that earlier in pregnancy. Postnatal exposure to dexamethasone in the first week of life permanently reduces hippocampal GR (but interestingly not MR) levels with no effect on basal corticosterone secretion (Felszeghy et al., 1996), providing further evidence that the effect of the programming stimulus depends on the time of exposure. Animals exposed to dexamethasone during the third trimester show hypertension and increased basal plasma corticosterone levels in adulthood (Levitt et al., 1996). They also show impaired learning and increased anxiety (Welberg et al., 2001), as do the offspring of stressed dams (Vallee et al., 1997) which also show impaired post-stress HPA axis feedback (Henry et al., 1994). The observed increase in anxiety may be a consequence of elevated CRH expression in the amygdala (Welberg et al., 2001).

"Neonatal handling" of rat pups has profound programming effects. In this paradigm, rat pups are subjected to 15 minutes daily maternal separation during the first 3 weeks of life (Meaney et al., 1985). In fact, the effects of handling were later found to be due to altered maternal behaviour, with increased licking and grooming of handled pups being induced by the brief separation (Liu et al., 1997). Adult handled animals showed increased hippocampal GR compared to nonhandled controls (Meaney et al., 1985; Meaney and Aitken, 1985). They also secreted less ACTH and corticosterone in response to stress and terminated corticosterone secretion more quickly following stress than did the nonhandled rats (Meaney et al., 1988; Meaney et al., 1989). The offspring of high licking/grooming dams showed similar changes in hippocampal GR mRNA expression and HPA axis activity to handled animals (Liu et al., 1997). Handled animals also showed increased GR mRNA and protein in the hippocampus and frontal cortex in old age (Meaney et al., 1985; Sarrieau et al., 1988) and maintained lower basal corticosterone levels, showed reduced hippocampal cell loss and had improved spatial memory compared to nonhandled controls (Meaney et al., 1988). Thus the effects of handling or high licking/grooming

seem to be to reduce the cumulative exposure of these animals to glucocorticoids over their lifespan and perhaps to prevent the deleterious effects of glucocorticoids during aging (section 1.9.2.6).

Interestingly, prolonged maternal separation (for 3h daily for the first 3 weeks of life or 24h once at postnatal days 3-4) seems to have the opposite effect to neonatal handling. Adult maternally-deprived animals showed reduced GR mRNA in their frontal cortex, hypothalamic paraventricular nucleus and hippocampal CA1 subfield (Avishai-Eliner et al., 1999), elevated ACTH levels in response to mild stress (Plotsky and Meaney, 1993) and may have impaired spatial memory in old age (Meaney et al., 1996) compared to nonseparated control animals. Unlike handled animals that show reduced anxiety, separated animals show normal anxiety responses to novelty (Biagini et al., 1998). These data suggest that maternally separated animals may be less able to "cope" with stress.

The overall effect of handling is proposed to be that the early postnatal environment increases glucocorticoid receptor gene expression, leading to increased sensitivity to the feedback effects of glucocorticoids, thus altering the responsivity of the axis to subsequent stressors (reviewed in (Meaney et al., 1996)). The mechanisms underlying the handling effect are not fully understood. The current model proposes that increased levels of circulating thyroxine and its metabolite triiodothyronine in handled pups lead to increased hippocampal 5-HT turnover, activation of hippocampal 5-HT receptors which increase intracellular cAMP leading to changes in GR expression (Meaney et al., 1987; Meaney et al., 2000; Mitchell et al., 1990a; Smythe et al., 1994). This hypothesis is supported by studies showing that *in vivo* administration of the 5-HT receptor antagonist ketanserin or the 5-HT neurotoxin 5,7-DHT decreased the handling effect on GR expression (Mitchell et al., 1990b). Also, 5-HT increased cAMP levels and subsequently GR levels in primary hippocampal cultures (Mitchell et al., 1990b; Mitchell et al., 1992) but not in glial cells (Mitchell et al., 1990a), while hippocampal cAMP is increased in rat pups immediately post-handling (Meaney et al., 1996). Finally, treatment of cultured hippocampal cells with the stable cAMP analogue 8-bromo-cAMP increases GR levels (Mitchell et al., 1992). However, there is as yet no direct *in vivo* evidence that

the effects of handling on GR expression are via 5-HT receptors activating the cAMP second messenger cascade. The mechanism by which GR gene expression is altered by handling is unknown, but recent work from this laboratory suggests that changes in alternate promoter usage in the GR gene may be involved, since neonatal handling caused a selective increase in the expression of GR mRNA transcripts containing the hippocampus-specific exon 1_7 (McCormick et al., 2000). This change in promoter usage may be via cyclic AMP-protein kinase A pathways involving the activation of NGFI-A and AP-2 (Meaney et al., 2000). It has been recently proposed that increased expression of these factors after handling might protect areas of the GR gene promoter from methylation during development, thus increasing their transcriptional activity in later life (Weaver et al., 2001). However, this is highly speculative.

During development, the expression of many receptors is influenced by the first encounter of receptor and ligand, a phenomenon termed "hormonal imprinting" (Csaba and Inczefi-Gonda, 1998; Gaal and Csaba, 1998). Also, perinatal exposure to molecules similar to the specific ligand can cause receptor binding and "false imprinting". GR is thus by no means unique, since the programming effect of prenatal dexamethasone on hepatic GR expression may be considered hormonal imprinting (Nyirenda et al., 1998) and GR is also subject to false imprinting by *in utero* exposure to the pollutant benzpyrene and neonatal administration of vitamin A, which permanently reduce and increase thymic GR levels respectively (Csaba et al., 1991; Csaba and Inczefi-Gonda, 1992; Gaal and Csaba, 1998). As seen above for perinatal programming of GR expression, the mechanism by which hormonal imprinting affects receptor expression is unknown. Like programming, however, imprinting is thought to involve postreceptor events (Csaba and Inczefi-Gonda, 2001).

1.8 Transgenic models of altered glucocorticoid action

A number of transgenic mouse models have been generated to help elucidate the functions of glucocorticoids in various tissues. In this section I shall summarise the various models and the phenotype associated with each. The relevance of particular

aspects of the phenotype in different organ systems will be highlighted in subsequent sections. The structure of the GR gene is described in section 1.6.

Some of the transgenic models alter the endogenous GR gene in all or a subset of tissues, while others were generated by addition of a transgene.

1.8.1 $GR^{hypo/hypo}$ mice

These mice were generated by insertion of a neomycin resistance cassette into exon 2 of the GR gene (Cole et al., 1995b). Mice homozygous for this mutant allele show no detectable GR mRNA by Northern blotting (Cole et al., 1995b) and a variety of phenotypic features consistent with abolished GR signalling. The majority of these mice die within a few hours of birth due to lung atelectasis (Cole et al., 1995b; Cole et al., 1995a).Also, they exhibit impaired HPA axis feedback regulation, increased plasma ACTH and corticosterone concentrations, reduced induction of glucocorticoid-induced enzymes (glucose-6-phosphatase, tyrosine aminotransferase and phosphoenolpyruvate carboxykinase) in the liver at birth and abnormal adrenal gland development with a lack of medullary chromaffin cells (Cole et al., 1995b; Cole et al., 1995a).

Despite these abnormalities, up to 20% of these mice survive to adulthood (Finotto et al., 1999) and, despite a lack of detectable GR mRNA and protein, surfactant levels in the lungs of these survivors are normal (Cole et al., 1995a). These data suggested that some GR activity persisted and it was subsequently discovered that the mice express mRNAs encoding an amino-terminal truncated GR protein containing the DNA and ligand-binding domains (Tronche et al., 1998). In fact, these mice show high affinity binding of dexamethasone in protein extracts of liver, kidney, lung and brain at levels 30-60% of those in wild-type mice (Cole et al., 2001). However, thymocytes and cultured hepatocytes from these animals remain dexamethasone-insensitive (Cole et al., 2001).

1.8.2 $GR^{null/null}$ mice

These mice have a complete lack of GR activity due to deletion of exon 3 of the GR gene which encodes the first zinc finger of the DNA binding domain (Finotto et al.,

1999). The phenotype of these mice is similar to that of the $GR^{hypo/hypo}$ mice but with increased penetrance and severity: 100% of mice homozygous for this allele die at birth due to lung atelectasis (Finotto et al., 1999) and show reduced gluconeogenic enzyme expression in the liver, a lack of glucocorticoid-mediated T cell apoptosis *in vitro*, impaired proliferation of erythroid progenitors (Tronche et al., 1998) and impaired stress erythropoiesis (Bauer et al., 1999).

Subsequently, a modified strategy placing the *Cre* recombinase under the control of the neuron-specific nestin promoter allowed the generation of mice (GR^{NesCre} mice) with a brain-specific GR knockout (Tronche et al., 1998; Tronche et al., 1999). These mice have no detectable GR in brain but normal pituitary GR (Tronche et al., 1999). Phenotypically, the mice show impaired HPA axis feedback, with increased CRH peptide levels in the hypothalamus, increased ACTH and POMC mRNA in the anterior pituitary and increased basal plasma ACTH and corticosterone levels (Tronche et al., 1999). They also show reduced growth rates, an increased incidence of osteoporosis and reduced anxiety responses (Tronche et al., 1999). Adult GR^{NesCre} mice also have disturbed energy balance, with reduced fat and protein deposition, reduced food intake and reduced metabolic efficiency which is possibly accompanied by disturbed control of circulating leptin levels (Kellendonk et al., 2002).

1.8.3 $GR^{dim/dim}$ mice

A point mutation altering a single amino acid (Ala458 to Thr) in the D loop of the GR protein disrupts the formation of GR homodimers (section 1.6). This mutation was engineered into the endogenous GR gene in mouse embryonic stem cells using a *Cre/LoxP* targeting strategy (Reichardt et al., 1998).

Transgenic mice homozygous for this allele have dimerisation-deficient GR but survive to adulthood and have normal fertility (Reichardt et al., 1998). There is a loss of transactivation associated with the DNA binding of GR dimers e.g. of the tyrosine aminotransferase gene, although the repressing function of GR via direct protein-protein interactions with other transcription factors (section 1.11.2) is retained (Reichardt et al., 1998). CRF expression in these animals is normal, but they exhibit increased pituitary POMC and ACTH expression (though serum ACTH

concentrations are normal) and increased basal serum corticosterone concentrations (Reichardt et al., 1998). Development of the adrenal medulla is normal (Reichardt et al., 1998), but thymocytes from these animals are completely resistant to dexamethasone (section 1.9.5.2).

These mutant receptors retain some ability to activate a mouse mammary tumour virus promoter *in vitro* (Heck et al., 1994; Reichardt et al., 1998). The normal development of the adrenal glands and lungs (section 1.9) of these mice (Reichardt et al., 1998) and their survival to adulthood suggests that such activation is also occurring *in vivo*, perhaps through cross-talk of the GR with other transcription factors (Reichardt et al., 1998). However, dimerisation of liganded GR monomers could occur via protein-protein interactions between their ligand-binding domains, so some dimerisation activity may still be present in these mice. Thus genes with high-affinity GREs in these mice may still be activated at low levels due to binding of GR monomers to GRE half-sites.

1.8.4 GR^{AS} mice

These mice express a 1.8kb GR antisense mRNA under the control of the neurofilament gene promoter (Pepin et al., 1992b). This promoter was originally intended to target expression of the antisense mRNA to the central nervous system, but this was unsuccessful: the mice in fact express the transgene in all tissues examined (Pepin et al., 1992b).

Transgenic mice exhibit a variable reduction in GR mRNA expression in all tissues studied, with a 50-70% reduction in the hypothalamus and frontal cortex and a 30-55% reduction in the liver. They also exhibit reduced GR binding sites in frontal cortex, hypothalamus, pituitary and liver (Pepin et al., 1992b). From this data it can be seen that this model is by no means a complete GR knockout. Grossly, the mice are much larger than control animals, with increased fat deposition but, interestingly, decreased appetite (Pepin et al., 1992b). The mice show HPA axis dysregulation with increased plasma ACTH and corticosterone levels (Pepin et al., 1992b).

1.8.5 $GR^{AS(Thy)}$ mice

These mice show thymus-specific expression of a GR antisense transgene and were also generated using a GR antisense mRNA construct, but expression of the transgene was restricted to thymocytes by placing it under the control of the *lck* promoter (King et al., 1995) which is only active in immature thymocytes (Garvin et al., 1990). Indeed, expression of the transgene was only detected in the thymus and at very low levels in the spleen (King et al., 1995). Phenotypically, these mice exhibit normal HPA axis activity and plasma corticosterone levels, a 50% reduction in GR mRNA and 43% reduction in GR protein in the thymus, normal GR mRNA levels in splenocytes, impaired glucocorticoid-mediated gene induction in thymocytes and altered thymocyte selection (section 1.9.5.2) (King et al., 1995).

Pazirandeh *et al* independently generated mice expressing antisense and sense GR transgenes in the thymus by the same technique (Pazirandeh et al., 2002). The mice expressing the antisense transgene show a 25% reduction in thymocyte GR, while those expressing the sense transgene show a twofold increase in thymocyte GR (Pazirandeh et al., 2002). Both transgenics show altered thymocyte selection, thymocyte numbers and thymus size (Pazirandeh et al., 2002).

1.8.6 Mice overexpressing GR from a YAC transgene (YGR mice)

These mice were created by microinjection of a 290kb yeast artificial chromosome (YAC) carrying 2 additional copies of the GR allele into mouse oocytes (Reichardt et al., 2000b). The mice showed a variable increase in GR mRNA expression in different tissues, with the highest overexpression in the pituitary and brain (43% and 60% respectively) and a 20-24% overexpression in spleen, thymus and liver (Reichardt et al., 2000b). However, in all tissues expression of the transgene is exactly equivalent to that of the endogenous GR gene, demonstrating autoregulation of the transgene by glucocorticoids (Reichardt et al., 2000b). YGR mice had approximately 50% more GR in the hippocampus than wild-type controls, indicating that the increase in GR mRNA expression was followed by an increase in GR protein levels (Reichardt et al., 2000b).

Phenotypically, these mice showed reduced basal corticosterone levels, increased basal ACTH levels, reduced responses to restraint stress, faster resolution of the stress response and reduced inflammatory responses compared to wild-type controls (Reichardt et al., 2000b).

1.9 The physiological role of glucocorticoids and GR

1.9.1 Glucocorticoids and development

Glucocorticoids influence many aspects of foetal development. Glucocorticoid synthesis begins early in the development of the foetal adrenal, with circulating levels rising to a peak at birth (Arai and Widmaier, 1993; Nagaya et al., 1995).

Glucocorticoids are essential for lung development: $GR^{null/null}$ mice (section 1.8.2) die at birth due to lung atelectasis (Finotto et al., 1999) and $CRH^{-/-}$ mice develop cyanosis and die within 24 hours of birth (Muglia et al., 1999). GR mRNA is mainly expressed in the lung mesenchyme, although there is some expression in the bronchial epithelium. Glucocorticoids promote the development of normal lung architecture: dexamethasone increases alveolar lumen size and type II (surfactant-producing) pneumocyte density in human foetal lung organ cultures (reviewed in (Mendelson, 2000)). This may be a partially indirect effect, by inducing the expression of mesenchymal factors that act on the epithelium to regulate morphogenesis and function (Mendelson, 2000). Glucocorticoids also promote surfactant synthesis. The synthetic glucocorticoid betamethasone increased the intracellular concentration of phosphatidyl glycerol and fatty acids (lipid activators of cholinephosphate cytidyltransferase, the rate-limiting enzyme in surfactant synthesis) (Mallampalli et al., 1994) and increased the expression of surfactant-proteins A, B and C (Ballard et al., 1996; McCormick and Mendelson, 1994).

The chromaffin cells of the adrenal medulla synthesise the catecholamines noradrenaline and adrenaline (Ganong, 1989). Glucocorticoids stimulate the differentiation of neural crest cells into a chromaffin rather than a neural phenotype and prevent neural crest cells developing a neural phenotype by reducing the expression of neural-specific genes e.g. SCG10 (Stein et al., 1988). Also, they induce expression of the enzyme phenylethanolamine-N-methyl transferase (PNMT), which

converts noradrenaline into adrenaline, in neural crest cells that are destined for a chromaffin phenotype (Jiang et al., 1989; Michelsohn and Anderson, 1992; Ross et al., 1990; Teitelman et al., 1982) but not those that are destined for a neuronal phenotype (Jiang et al., 1989). Perplexingly, despite the fact that $GR^{hypo/hypo}$ mice (section 1.8.1) have a disorganized adrenal medulla and chromaffin cells containing reduced levels of PNMT (Cole et al., 1995b; Cole et al., 1995a), $GR^{null/null}$ mice have normal chromaffin cell numbers (though they lack PNMT expression) (Finotto et al., 1999). It has been suggested that glucocorticoid signaling may only modulate chromaffin cell differentiation with spatial restriction of biochemical signals responsible for stabilizing the neuronal phenotype, adrenal inhibitors of neuronal differentiation or neurite-repulsive cues reaching chromaffin cells being mainly responsible for differentiation (Finotto et al., 1999).

1.9.2 Central effects of glucocorticoids

Glucocorticoid receptors are widely expressed in the brain (reviewed in (McEwen et al., 1986)). GR mRNA is particularly abundant in the hippocampus, with highest levels in CA1/2 and dentate gyrus and lower levels in CA3 (Herman et al., 1989a; Sousa et al., 1989). The distribution of GR binding sites (Gerlach and McEwen, 1972; Sapolsky et al., 1983) and receptor protein (Fuxe et al., 1985) in the brain and hippocampus is consistent with the mRNA distribution. Evidence suggests that hippocampal GR mRNA levels vary dynamically during intrauterine development, with highest levels during the third trimester of pregnancy (Andrews and Matthews, 2000).GR is also abundant in the cerebellum, especially during development (Pavlik and Buresova, 1984), amygdala, cortex, thalamus and hypothalamus (Sousa et al., 1989).

The mineralocorticoid receptor is also expressed throughout the brain (Arriza et al., 1988) but high levels of expression are mainly restricted to the lateral septum and hippocampus (Arriza et al., 1988; deKloet et al., 1975; Reul and DeKloet, 1985). In the hippocampus, MR mRNA is most abundant in the proximal part of CA3, followed by CA1/2 and dentate gyrus with lower levels in the distal part of CA3 (Herman et al., 1989a). MR mRNA is more abundant than GR mRNA in all

hippocampal subfields (Herman et al., 1989a). The distribution of MR protein in the hippocampus is largely similar to that of MR mRNA with highest levels in CA1 and dentate gyrus (Reul and DeKloet, 1985). Due to absence of 11β-HSD2 (Robson et al., 1998), the MR in hippocampus effectively function as glucocorticoid receptors.

1.9.2.1 Central control of glucocorticoid secretion

The secretion of glucocorticoids is controlled by the activity of the hypothalamic-pituitary-adrenal (HPA) axis (Figure 1.6) via a classical negative feedback loop. Glucocorticoid secretion follows a circadian rhythm, with the greatest secretion being just before the beginning of the animal's period of activity (in the morning in humans and in the evening in rats). This "clock" is ultimately governed by the suprachiasmatic nucleus of the hypothalamus. Also, glucocorticoid secretion increases as part of the stress response (section 1.9.3).

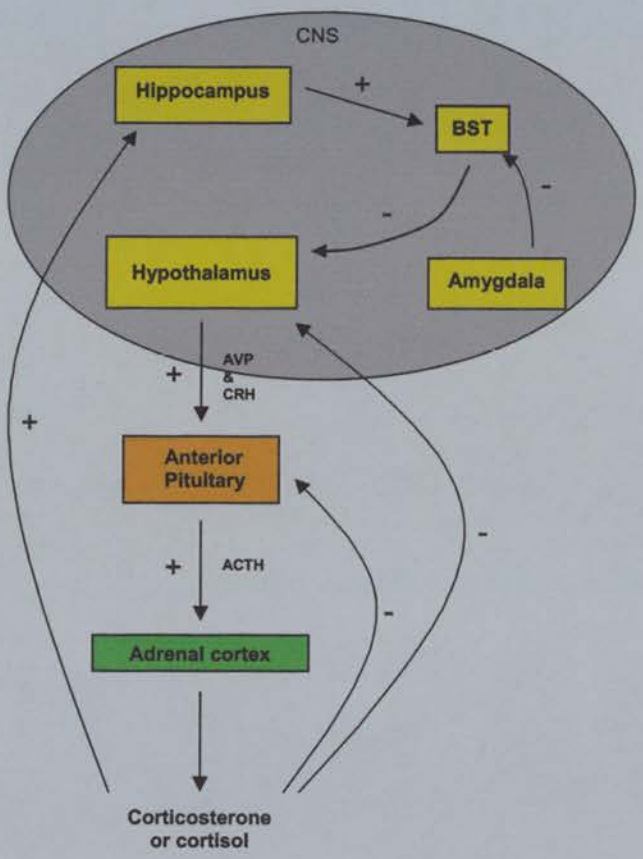


Figure 1.6: the hypothalamic-pituitary-adrenal axis. Adapted from (Ganong, 1989).

1.9.2.2 Pituitary control of adrenal activity

Glucocorticoid production in the adrenal cortex is stimulated by adrenocorticotrophic hormone (ACTH), a 39 amino acid polypeptide produced in the anterior pituitary from the prohormone proopiomelanocortin (POMC). POMC is the precursor for several other hormones (including as ß-lipotropin, an endorphin/encephalin precursor) and is secreted from several other tissues e.g. the hypothalamus, lungs, gastrointestinal tract and placenta (Ganong, 1989).

ACTH rapidly stimulates glucocorticoid synthesis by activating the cyclic AMP (cAMP) second messenger system via a high-affinity G-protein coupled membrane receptor. Increased intracellular cAMP activates protein kinase A, which in turn phosphorylates cholesterol ester hydrolase thus increasing conversion of cholesterol esters to free cholesterol (Ganong, 1989).

Prolonged exposure to ACTH increases the maximum adrenal secretory capacity by delayed induction of the 5 cytochrome P_{450} enzymes involved in glucocorticoid synthesis (Ganong, 1989) through a poorly-defined signalling cascade (Parker and Schimmer, 1993).

Corticotrophin-releasing hormone (CRH, a 41 amino acid polypeptide) and arginine vasopressin (AVP) secreted from projections of the parvocellular neurones of the medial hypothalamic paraventricular nucleus travel through the hypophyseal portal circulation to the anterior pituitary where they stimulate POMC production and ACTH release (Ganong, 1989). Various other peptides e.g. vasoactive intestinal polypeptide, pituitary AC-activating polypeptide, secretin, glucagon, glucagon-like peptide, parathyroid hormone and parathyroid hormone related protein may modulate HPA axis activity (reviewed in (Nussdorfer et al., 2000)).

1.9.2.3 Feedback regulation at the hypothalamus and pituitary

Much evidence supports the hypothalamus as a site of negative feedback via GR, which is abundant in this area as discussed above. Glucocorticoids regulate CRH mRNA expression in hypothalamic parvocellular neurons (Herman et al., 1992),

possibly via GR binding to a negative GRE (Malkoski et al., 1997). Lesions of the ventromedial hypothalamus disrupt glucocorticoid negative feedback ((Suemaru et al., 1995), reviewed in (McEwen et al., 1986)). Furthermore, studies using the chronic hypovolaemia stress model suggest that corticosterone levels mediate CRH secretion in 2 ways, with inhibition via the GR and facilitation of secretion via MR-mediated signaling which functions to maintain transcription in the face of chronic activation (reviewed in (Tanimura and Watts, 2001)). More recently, GR^{NesCre} mice (section 1.8.2) showed HPA axis hyperactivity with a 5-fold increase in CRH production (Kretz et al., 1999), increased POMC expression in the anterior pituitary and increased plasma corticosterone concentration (Tronche et al., 1998) consistent with impaired glucocorticoid feedback on HPA axis activity. Conversely, YGR mice show a more than twofold reduction in CRH immunoreactivity in the median eminence of the hypothalamus (Reichardt et al., 2000b).

The pituitary has been long established as a site of glucocorticoid negative feedback (Ganong, 1989) which occurs by fast, intermediate and slow mechanisms (reviewed in (Keller-Wood and Dallman, 1984)). The fast and intermediate mechanisms do not require changes in gene transcription (reviewed in (Keller-Wood and Dallman, 1984)) and fast feedback was unaffected by the protein synthesis inhibitor actinomycin D, supporting the hypothesis that it occurs via a nongenomic mechanism (Hinz and Hirschelmann, 2000). The slow mechanism involves the classical genomic steroid mechanism of action (reviewed in (Keller-Wood and Dallman, 1984)); POMC gene transcription and thus ACTH production in the anterior pituitary are reduced by glucocorticoids (Drouin et al., 1987; Seger et al., 1988), probably via a negative GRE (Drouin et al., 1987; Riegel et al., 1991). Also, dexamethasone decreases CRF receptor mRNA in cultured rat anterior pituitary cells (Sakai et al., 1996).

GR is important in pituitary glucocorticoid feedback. Immuntohistochemistry located numerous glucocorticoid receptors in the anterior pituitary (Antakly and Eisen, 1984). Dexamethasone suppression of ACTH secretion has been shown to act via pituitary GR (Cole et al., 2000) and pituitary adenomas that were unresponsive to dexamethasone suppression had significantly lower GR mRNA than glucocorticoid-

responsive tumours (Mu et al., 1998). Administration of the GR antagonist RU486 to patients with pituitary-dependent Cushing's Disease caused an increase in circulating cortisol while the same treatment had no effect on patients with non-pituitary Cushing's Disease (Bertagna et al., 1986). $GR^{hypo/hypo}$ mice show a 20-fold increase in plasma ACTH and a 2-3 fold increase in plasma corticosterone compared to normal mice (Cole et al., 1995a) while $GR^{dim/dim}$ mice exhibit elevated POMC mRNA levels and a 2.2-fold increase in ACTH immunostaining in their pituitary, indicating that feedback inhibition of POMC/ACTH may be abolished at the transcriptional level in these animals (Reichardt et al., 1998). However, these animals do not show elevated serum ACTH levels, attributed by the authors to a DNA-binding independent regulation of ACTH secretion by GR (Reichardt et al., 1998). Conversely, YGR mice show an approximately threefold reduction in POMC and ACTH expression in the anterior pituitary, with a fourfold reduction in serum corticosterone levels compared to wild-type mice (Reichardt et al., 2000b). Perplexingly, these mice also show elevated plasma ACTH levels, attributed to developmental influences causing a new equilibrium of basal hormone secretion due to GR overexpression (Reichardt et al., 2000b).

Inactivation of GR in the nervous system of GR^{NesCre} mice allows discrimination between the function of GR in the brain and anterior pituitary (Tronche et al., 1999). Despite normal GR-mediated negative feedback in the anterior pituitary, these mice exhibit elevated basal glucocorticoid levels due absent PVN negative feedback (Tronche et al., 1999) which disinhibits CRH production, leading to increased ACTH synthesis in the anterior pituitary (Tronche et al., 1999). However, circulating ACTH levels are slightly reduced, possibly due to the increased corticosterone levels inhibiting pituitary ACTH secretion (Tronche et al., 1999). The data from the $GR^{dim/dim}$, YGR and GR^{NesCre} mice shows that changes in circulating glucocorticoid levels are not always accompanied by a parallel change in ACTH levels. Also, the absence of GR in the brain leads to an increase in HPA axis activity which pituitary GR cannot compensate for (Tronche et al., 1999).

Mice ubiquitously expressing a GR antisense transgene exhibit signs of HPA axis hyporesponsiveness to glucocorticoids, requiring 10x higher doses of dexamethasone

to completely suppress corticosterone secretion than controls (Barden et al., 1997; Stec et al., 1994). Furthermore, the mice show a shorter duration of dexamethasone suppression of ACTH secretion (Barden et al., 1997), enhanced ACTH responses to stress (Karanth et al., 1997) and reduced corticosterone negative feedback on CRH release (Karanth et al., 1997) compared to controls. However, there is some controversy over the level of basal HPA axis activity in these animals, with initial findings of increased plasma ACTH and corticosterone concentrations (Pepin et al., 1992b) being rebutted in a later report (Karanth et al., 1997), suggesting that the high levels measured initially may not have been representative.

1.9.2.4 Other central influences on HPA axis activity

The hypothalamus receives neuronal inputs from several brain regions and integrates a variety of complex environmental and emotional influences on HPA axis activity, e.g. fear (via the amygdala, see below), diurnal rhythm (from the suprachiasmatic nucleus), nociceptive stimuli (via the reticular formation) and baroceptor inputs (from the nucleus tractus solitarius) (Ganong, 1989).

The hypothalamus also receives indirect neural inputs from the hippocampus via inhibitory GABAergic projections from the bed nucleus of the stria terminalis (BST) and the preoptic nuclei (reviewed in (Herman and Cullinan, 1997)). The hippocampus has a complex role in HPA axis regulation that is still the subject of some controversy. Total or dorsal hippocampectomy causes increased CRF and AVP expression in the hypothalamic paraventricular nucleus and increases circulating corticosterone levels (Herman et al., 1989b). Also, inhibition of hippocampal GR and MR using implanted antagonist pellets attenuated the inhibitory effect of dexamethasone on CRH release from the hypothalamus and increased the CRH response to auditory and photic stimulation (Feldman and Weidenfeld, 1999). These data support the hypothesis that the hippocampus has an inhibitory influence on HPA activity (reviewed in (Jacobson and Sapolsky, 1991)).

Both types of glucocorticoid receptor, MR and GR, are expressed in the hippocampus (Herman et al., 1989a; McEwen et al., 1986). The MR in brain bind

corticosterone with high affinity but low capacity, so are largely occupied under basal conditions, whereas the GR bind corticosterone with lower affinity but higher capacity (as there are more GR present than MR) and thus become occupied at higher corticosterone levels e.g. during stress (Reul et al., 1987b; Spencer et al., 1990). Indeed, under conditions of basal HPA axis activity the majority of glucocorticoid signalling in the hippocampus seems to be mediated by MR signalling, since adrenalectomy-induced elevations in ACTH secretion are suppressed by corticosterone but not by the predominantly GR ligand dexamethasone (Kovacs and Makara, 1988) and intracerebroventricular administration of a MR antagonist increases plasma corticosterone levels at the morning nadir in rats (Ratka et al., 1989). Studies using the MR antagonist spironolactone in rats (Bradbury et al., 1994) and humans (Deuschle et al., 1998) again suggest that MR activation controls HPA axis activity at the diurnal trough in glucocorticoid secretion. Interestingly, there appears to be some cross-talk between the MR and GR signaling pathways since hippocampal MR signaling appears to potentiate the inhibition of ACTH secretion by GR activation (Bradbury et al., 1994).

However, hippocampal GR also seem to be important in controlling HPA axis activity, especially at higher glucocorticoid levels e.g. during stress or at the diurnal peak of glucocorticoid secretion. Chronic stress causes downregulation of hippocampal GR over 7 day (Paskitti et al., 2000), 14 day (Kitraki et al., 1999), 18 day (Kim et al., 1999), 3 week (Sapolsky et al., 1984b) and 4 week (Mizoguchi et al., 2001) experimental periods. This effect is likely to be partly mediated by glucocorticoids (section 1.7.2.1), but probably involves signalling via several neurotransmitter systems as discussed in section 1.7.2.2. Depletion of hippocampal GR causes corticosterone hypersecretion after stress, but leaves basal corticosterone levels unchanged (Sapolsky et al., 1984a). At glucocorticoid levels above basal, the GR antagonist RU38486 decreases ACTH secretion (van Haarst et al., 1997), but has no effect on basal corticosterone secretion (Ratka et al., 1989). Evidence from $GR^{dim/dim}$ mice suggests that the actions of corticosteroids in the hippocampus require the DNA binding of GR homodimers (Karst et al., 2000) and YGR mice show a 60% increase in hippocampal GR, reduced CRH, POMC and ACTH levels and a fourfold

reduction in basal serum corticosterone levels (Reichardt et al., 2000b). Additionally, manipulations that decrease hippocampal GR are consistently associated with diminished feedback regulation and elevated plasma glucocorticoid levels (Henry et al., 1994; Jacobson and Sapolsky, 1991) whereas manipulations that increase hippocampal GR have the opposite effect (Meaney et al., 1988; Meaney et al., 1989; Meerlo et al., 1999; O'Donnell et al., 1994). However, it has been suggested that increased hippocampal GR might indirectly improve feedback regulation by altering behaviour and the animal's ability to cope with stress ((Vallee et al., 1997), reviewed in (Anisman et al., 1998)).

As mentioned above, other brain regions can influence hypothalamic and hence HPA axis activity. Like the hippocampus, the medial prefrontal cortex (MpFC) has inhibitory inputs to the hypothalamus via the BST and preoptic nuclei (Herman and Cullinan, 1997) and lesions of the MpFC potentiate the increase in ACTH and plasma corticosterone after restraint stress in rats (Diorio et al., 1993), suggesting that it modulates glucocorticoid inhibition of stress-induced HPA axis activity.

Data from studies using direct administration of adrenergic and serotonergic agonists to the amygdala (Feldman et al., 2000) suggest that the amygdala stimulates HPA axis activity by increasing CRH release from the hypothalamus by increasing noradrenergic and serotonergic neurotransmission (Feldman et al., 1987; Feldman et al., 1988). This effect is blocked by neurotoxic lesions of the ventromedial hypothalamus (Feldman et al., 2000) and HPA axis responses to stimulation of the amygdala are sensitive to glucocorticoid negative feedback (Weidenfeld et al., 1997). As well as its neural connections to the hypothalamus, the amygdala inhibits outflow from the BST (reviewed in (Raber, 1998)). Thus during stress the amygdala increases HPA axis activity and strengthens fear memory (Raber, 1998).

Endogenous opioid peptide systems may also modulate HPA axis responses to stress. Opioid receptors are ubiquitously expressed in the CNS and opioids can modulate the release of CRH from the hypothalamic parvocellular neurons (reviewed in (Drolet et al., 2001)).

1.9.2.5 Neuronal development and survival

In several mammalian species the dentate gyrus of the hippocampus develops new granular neurons in adulthood. Evidence from studies in rodents and primates suggests that these late-developing neurons may have an important role in hippocampal function (reviewed in (Gould and Tanapat, 1999)). For example, training in a task that requires hippocampal function for acquisition results in an increase in the number of adult-generated granule cells (Gould et al., 1999).

Glucocorticoids influence this process of adult neurogenesis: experimental elevations of corticosterone in adulthood reduce dentate gyrus neurogenesis (Cameron and Gould, 1994) and removal of adrenal steroids stimulates the proliferation of granule cell precursors (Cameron and McKay, 1999). Since GR and MR are absent from most adult granule cell precursors (Cameron et al., 1993) this effect is probably via glutamate receptor mediated excitatory inputs, since blockade of glutamate receptors enhances granule cell precursor proliferation (Gould et al., 1994) and activation of glutamate receptors suppresses it (Cameron et al., 1995). However, MR knockout animals show impaired granule cell neurogenesis and decreased density of granule cells, suggesting that MR signaling may have long-term trophic effects on these cells (Gass et al., 2000). Glucocorticoids also regulate the expression of neurotrophic factors in the hippocampus (Chao et al., 1998b), which may influence neurogenesis.

Perplexingly, glucocorticoids seem to be both neurotoxic and neuroprotective. Glucocorticoids are essential for neuronal survival: both acute (Gould et al., 1990b; Hu et al., 1997) and chronic (Sloviter et al., 1989) adrenalectomy cause loss of hippocampal granule cells. The acute loss is associated with decreases in cell body area and dendritic branching (Gould et al., 1990b) and can be prevented by corticosterone replacement (Gould et al., 1990b) or aldosterone replacement (Woolley et al., 1991), suggesting that occupation of hippocampal MR is sufficient to prevent cell loss. The dying cells seem to undergo apoptosis, showing chromatin condensation, nuclear fragmentation, compaction of cytoplasm and pyknosis without inflammation (Sloviter et al., 1993a; Sloviter et al., 1993b). Indeed, apoptosis may

represent a common pathway for glucocorticoid-mediated neuronal death (reviewed in (Reagan and McEwen, 1997)).

Glucocorticoids may also potentiate neuronal damage due to a variety of pathophysiological insults. Acute administration of dexamethasone has been shown to induce apoptosis in all regions of the hippocampus (Haynes et al., 2001) and adrenalectomy reduces ischaemic neuronal damage in the hippocampus (Sapolsky and Pulsinelli, 1985) and attenuates hippocampal neurotoxicity due to the glutamate receptor agonist kainic acid(Sapolsky, 1985). Conversely, corticosterone replacement potentiates both of these toxicities (Sapolsky, 1985; Sapolsky and Pulsinelli, 1985).

Furthermore, glucocorticoids may potentiate neurotoxicity due to excess neurotransmitter release. Exogenous corticosterone administration causes apical dendritic atrophy in CA3 pyramidal cells while leaving CA3 basal dendrites, CA1 pyramidal cells and dentate gyrus granule cells unaffected ((Woolley et al., 1990), reviewed in (McEwen, 1999)). However, CA3 pyramidal neurons have little GR compared to other hippocampal regions (Herman et al., 1989a; Sousa et al., 1989). The granule cells of the dentate gyrus are GR- and MR-rich (Reul and DeKloet, 1985) and increased glutaminergic neurotransmission from via their heavy mossy fibre projections to the CA3 pyramidal neurons (reviewed in (McEwen, 1999)) appears to cause the neurotoxic effect. Indeed, selective blockade of glutamate receptors prevents it (Magarinos and McEwen, 1995). This atrophy of CA3 neurons is reversible (reviewed in (Reagan and McEwen, 1997)). However, chronic exposure to elevated corticosterone levels can cause loss of CA3 neurons in rats (Sapolsky et al., 1985).

Glucocorticoids also influence hippocampal neuronal survival during aging. Aged rats show a decrease in CA3 pyramidal cell density (Landfield et al., 1981b). Adrenalectomy reduces this CA3 neuronal loss (Landfield et al., 1981a).

1.9.2.6 Glucocorticoids, cognition and memory

Glucocorticoid deficiency and excess can both affect cognition. Adrenalectomy causes memory impairment (Oitzl and DeKloet, 1992; Roozendaal et al., 1996),

which can be reversed by dexamethasone administration (Roozendaal et al., 1996). The detrimental effects of chronic stress or pathophysiological conditions (e.g. affective disorders) on cognition (reviewed in (McEwen and Sapolsky, 1995)) are probably due to glucocorticoid excess (Arbel et al., 1994; Bodnoff et al., 1995; Dachir et al., 1993). Furthermore, high levels of glucocorticoids impair memory retrieval in rats (de Quervain et al., 1998) and may be involved in the development of traumatic amnesia in human patients (Joseph, 1998).

Interestingly, moderate doses of glucocorticoids or GR agonists enhance memory when given immediately after training (Flood et al., 1978; Kovacs et al., 1977; Roozendaal et al., 1999; Roozendaal and McGaugh, 1996a; Roozendaal and McGaugh, 1996b; Roozendaal and McGaugh, 1997a) and brief periods of stress usually enhance the formation of new memories (Shors et al., 1992). The above evidence suggests that the effects of glucocorticoids on memory consolidation show an "inverted U" dose response, with both high and low levels causing memory impairment.

Excess glucocorticoids seem to affect cognition in two ways. Firstly, excess glucocorticoids may alter the electrophysiology of the hippocampus e.g. by impairing long-term potentiation (Dubrovsky et al., 1987; Pavlides et al., 1993) or increasing after-hyperpolarisation (Joels and DeKloet, 1989).

Secondly, they cause neuropathological changes, particularly in the hippocampus (section 1.9.2.1). The hippocampus has an important role in cognition, including declarative or episodic memory processes (required for event learning and recall), spatial memory and probably attention, arousal and emotional states (reviewed in (Lathe, 2001)). Patients with Cushing's syndrome and aged humans with significantly elevated cortisol levels show cognitive impairments consistent with hippocampal dysfunction (reviewed in (Belanoff et al., 2001; Brown et al., 1999; Sapolsky, 2000)). Also, cortisol hypersecretion may be implicated in dementia due to destruction of hippocampal neurons in Alzheimer's disease and hippocampal sclerosis (reviewed in (Belanoff et al., 2001)).

Several pieces of evidence suggest that the effects of glucocorticoids on cognition are mediated by GR. GR^{AS} mice and $GR^{hypo/hypo}$ mice (Oitzl et al., 1997) have impaired spatial memory and $GR^{hypo/hypo}$ mice show impaired memory consolidation (Oitzl et al., 1997). Also, pre or immediately post-training infusions of a GR but not an MR antagonist impair spatial memory in a water maze (Oitzl and DeKloet, 1992; Roozendaal et al., 1996), and GR binding capacity correlates with spatial memory performance following an inverted-U shaped curve while MR occupancy does not (Conrad et al., 1999).

Sapolsky has put forward the "glucocorticoid cascade hypothesis" to explain the hippocampal changes seen in aging and dementia .He has proposed that excessive glucocorticoid secretion causes downregulation of hippocampal GR and hippocampal neuron loss, reducing HPA axis negative feedback leading to further hypersecretion of glucocorticoids and more destruction of hippocampal neurons (Sapolsky et al., 1986). However, aged rats with impaired ability to perform in a water-maze test showed similar hippocampal GR and MR levels to young control animals, suggesting that age-related cognitive deficits are not necessarily related to altered hippocampal GR levels (Yau et al., 1994). Also, the hypothesis that reduced hippocampal GR function leads to impaired negative feedback, excessive glucocorticoid secretion and thus harmful effects via increased GR signalling appears paradoxical, since if GR levels are reduced then the amount of GR signalling should be reduced also. However, there are 3 possible explanations for the deleterious effects of increased cortisol levels in the face of reduced GR levels (reviewed in (McQuade and Young, 2000)). First, the increase in cortisol levels may be so great that it overcomes the reduction in GR capacity and increases GR signalling overall. Second, GR capacity in brain areas not associated with negative feedback may be normal, giving increased GR signalling in these areas. Third, the damaging effects of high cortisol may in part be via increased MR signalling (or a change in the balance of MR and GR signalling) or via non-receptor-mediated events.

The modulatory effects of glucocorticoids on cognition are also partly via GR in the basolateral amygdala: direct administration of GR agonists there enhances memory consolidation while administration of antagonists impairs it (Roozendaal and

McGaugh, 1997b). The enhancing effects of glucocorticoids on memory consolidation also depend upon the integrity of the amygdala β-adrenergic inputs from the locus coeruleus and nucleus of the solitary tract, whose cell bodies contain high densities of GR (reviewed in (Roozendaal, 2000)). The amygdala may integrate hormonal and neuromodulatory influences on memory consolidation in other brain structures, including the hippocampus. The basolateral amygdala projects to the hippocampus both directly and indirectly via the entorhinal cortex (Racine et al., 1983; Thomas et al., 1984). Lesions of the basolateral amygdala block the enhancing effect of post-training administration of glucocorticoids (Roozendaal and McGaugh, 1996a) and the detrimental effects of adrenalectomy on memory consolidation (Roozendaal et al., 1996): lesions of the stria terminalis (a major afferent-efferent pathway of the amygdala) have the same effects (Roozendaal and McGaugh, 1996b).

1.9.2.7 Glucocorticoids and mood disorders

Elevations of basal plasma cortisol levels associated with depression and other mood disorders have been well-documented (reviewed in (Ehlert et al., 2001; McQuade and Young, 2000; Pariante and Miller, 2001)). Compared with controls, people with depression show a loss of the normal circadian rhythm of cortisol secretion, with elevated nadir plasma cortisol concentrations (Deuschle et al., 1997). Furthermore, depressed patients show increased cerebrospinal fluid CRH concentrations (Nemeroff et al., 1984), suggesting that their hypercortisolaemia may be due to hypersecretion of CRH. Patients with unipolar and bipolar disorders show cortisol non-suppression in response to dexamethasone (Zhou et al., 1987), suggesting impaired glucocorticoid negative feedback on HPA axis activity. These effects are probably mediated via GR. GR^{NesCre} mice show reduced anxiety-related behaviour compared to wild-type mice (Tronche et al., 1999). Also, reduced GR mRNA is seen in the hippocampi of patients with the neuropsychiatric disorders mentioned above (Webster et al., 2000).

Glucocorticoids may also influence the pathogenesis of mood disorders by altering the activity of other neurotransmitter systems e.g. by altering $GABA_A$ receptor subtype composition in the hippocampus, which may alter GABAergic

neurotransmission (Cullinan and Wolfe, 2000). Disturbances of the 5HT system are consistently associated with depression (Meltzer, 1990) and long-term exposure to high doses of corticosterone reduces the responsiveness of rat hippocampal CA1 neurones to 5HT (Karten et al., 1999). Glucocorticoids appear to decrease the expression of the $5HT_{1A}$ (Meijer et al., 1997; Neumaier et al., 2000), $5HT_{2C}$ (Holmes et al., 1995a), $5HT_6$ and $5HT_7$ receptors (Yau et al., 1997a) in the hippocampus. This effect seems to be region and receptor subtype-specific, since the magnitude of changes in $5HT_{1A}$ expression with adrenalectomy and glucocorticoid replacement varies in different hippocampal subfields (Neumaier et al., 2000), $5HT_{2C}$ expression varies throughout the hippocampus with chronic arthritis stress and $5HT_6$ and $5HT_7$ expression is regulated by adrenalectomy only in CA1 and CA3 (Yau et al., 1997a). The effects of glucocorticoids on 5HT receptor expression appear to be mediated by both GR and MR (Cyr et al., 2001; Holmes et al., 1995a; Meijer et al., 1997; Neumaier et al., 2000). At least part of the effect of treatments used in mood disorders may be to normalise GR levels (section 1.7.2.3) and hence glucocorticoid secretion and 5HT receptor expression. Indeed, the tricyclic antidepressant desipramine reverses the HPA axis hyperactivity seen in a transgenic mouse model of the endocrine changes seen in depression (Pepin et al., 1992a).

1.9.2.8 Glucocorticoids and glial cells

The macroglia and microglia are a heterogeneous group of cell types that interact in many ways with neurones to influence their differentiation, development and metabolism (reviewed in (Garcia-Segura et al., 1996)), modulate potassium currents in neuronal membranes (Wu and Barish, 1994), regulate neuronal activity by regulating glucose metabolism (Pellerin and Magistretti, 1994) and maintain synaptic transmission in the hippocampus (Keyser and Pellmar, 1994). Glial cells express GR (Erdeljan et al., 2001; Morita et al., 1999) but not MR (Erdeljan et al., 2001) and interestingly glial cell GR does not seem to be regulated by glucocorticoids or serotonin (Erdeljan et al., 2001).

Glucocorticoids, presumably acting via GR, potentiate oligodendrocyte proliferation by regulating the expression of myelin basic protein, proteolipid protein and glycerol

phosphate dehydrogenase (Kumar et al., 1989; Tsuneishi et al., 1991), inhibit astroglia proliferation in vitro (Kniss and Burry, 1985) and mitosis *in vivo* (Gould et al., 1990a) and enhance death of glial cells deprived of serum (Morita et al., 1999). This last effect may be significant in ischaemic insults to the central nervous system. Neuroactive steroids may also be locally produced and metabolised by glia, which thus may modulate some of their central effects (reviewed in (Garcia-Segura et al., 1996)).

1.9.3 The stress response

Acute physical stress (such as illness, trauma or pursuit by a predator) or psychological stress (such as bereavement, public speaking or a mental illness) increases HPA axis activity and glucocorticoid secretion. This effect is essential in the short term to allow the animal to survive the stressor: the glucocorticoid release affects metabolism, enhancing energy availability, and also inhibits nonessential processes such as inflammation, growth and reproduction. Also, glucocorticoids protect the body from overreactivity of the immune system during illness (section 1.9.5.1). The stress response is terminated by the negative feedback effects of increased circulating levels of glucocorticoids on the HPA axis (section 1.9.2.2). This feedback control is crucial in preventing the detrimental effects of prolonged elevations of circulating glucocorticoid levels: these include myopathy, osteoporosis, hypertension, diabetes and insulin resistance, reproductive failure and infections (Ganong, 1989).

1.9.4 Glucocorticoids and intermediary metabolism

GR has an important role in metabolic control and is abundant in metabolically active tissues such as liver (Antakly and Eisen, 1984; Reul et al., 1989), muscle (Snochowski et al., 1980) and adipose tissue (Feldman and Loose, 1977). Overall, glucocorticoid release tends to raise blood glucose levels by increasing peripheral protein catabolism, hepatic uptake of amino acids, deamination and transamination of amino acids and decreasing peripheral and hepatic glucose utilisation (Ganong, 1989).

In the liver, GR levels vary with age. In mice, they are higher at 30d postnatally than at 10d or 60d (Borbhuiya and Sharma, 1995) and in rats they are greater in immature than mature animals (Sharma and Timiras, 1987). Aged rats also show reduced hepatic GR (Djordjevic-Markovic et al., 1999). Although hepatic expression of GR was initially thought to be homogeneous (Antakly and Eisen, 1984), recent evidence suggests that there are higher levels of GR mRNA in periportal hepatocytes than in perivenous hepatocytes (Nyirenda et al., 1998).

GR is central to control of hepatic carbohydrate metabolism, although glucocorticoids do interact with other hormones controlling intermediary metabolism (see below). $GR^{hypo/hypo}$ mice show impaired glucocorticoid-mediated induction of hepatic tyrosine aminotransferase and serine dehydrogenase mRNA expression (Cole et al., 1995b; Cole et al., 1995a), as do $GR^{dim/dim}$ mice (Reichardt et al., 1998). $GR^{dim/dim}$ mice also show impaired glucocorticoid-mediated induction of pyruvate carboxykinase mRNA expression (Reichardt et al., 1998). Furthermore, glucocorticoids have been shown to regulate the expression of several other enzymes of glycolysis and gluconeogenesis, such as 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase which controls the level of gluconeogenesis in the cell (reviewed in (Lemaigre and Rousseau, 1994)), glucose-6-phosphatase (Schmoll et al., 1999) and phosphoenolpyruvate carboxykinase (in insulin-resistant animals) (Friedman et al., 1993). Hepatocyte function varies between periportal and perivenous hepatocytes: this is the basis of the "metabolic zonation" model, according to which glycogenolysis, gluconeogenesis, amino acid utilization, ammonia detoxification, protective metabolism (glutathione peroxidation and conjugation), bile formation, and the synthesis of some plasma proteins such as albumin and fibrinogen occur mainly in the cells of the upstream or periportal area while glucose utilization, xenobiotic metabolism, and the formation of other plasma proteins occur mainly in the cells of the downstream or perivenous zone (reviewed in (Jungermann and Kietzmann, 1996)). Thus hepatic GR may be higher in the periportal hepatocytes colocalising with metabolic processes under glucocorticoid control, although this is as yet unproven.

GR signalling also affects the rate of lipid synthesis. In the liver, glucocorticoids increase glucose-6-phosphatase expression and hence increase the phosphorylation state of the cell, favouring lipogenesis (reviewed in (Berdanier, 1989)) and obese Zucker rats show increased in hepatic GR levels compared to lean controls (Jenson et al., 1996). Furthermore, GR^{AS} mice show greatly increased fat deposition compared to controls, GR^{NesCre} mice have reduced body fat compared to controls and normal rats starved then refed a high-glucose diet show a 2-3 fold induction of fatty acid synthesis, hepatic lipid content and hepatic lipogenic activity that is decreased by adrenalectomy and restored by glucocorticoid replacement (reviewed in (Berdanier, 1989)). The effects of glucocorticoids on lipid metabolism may be tissue-specific. Glucocorticoids affect the distribution of body fat; GR^{NesCre} mice showed redistribution of fat towards the head and neck (Tronche et al., 1999) and patients with Cushing's disease showed decreased peripheral and increased central deposition of fat (Ganong, 1989). This redistribution may be because glucocorticoids acutely activate lipolysis and increase free fatty acid release in peripheral adipose tissue (Orth, 1998).

As mentioned above, glucocorticoids interact with other hormones controlling intermediary metabolism. In cultured rat hepatocytes insulin receptor binding is increased by dexamethasone in a dose-dependent manner (Fleig et al., 1985) and insulin replacement in diabetic rats increases hepatic GR levels (Yourick and Beuving, 1985). Glucocorticoids potentiate induction of glucokinase expression after refeeding (Minderop et al., 1987) and insulin inhibited glucocorticoid induction of 6-phosphofructo-2-kinase (Pierreux et al., 1998). Furthermore, GR may interact directly with other (liver-specific) transcription factors e.g. HNF-4 and HNF-3 to modulate hepatic gene expression and influence metabolic processes (reviewed in (Desimone and Cortese, 1992)): the possible mechanism of such interactions is discussed in section 1.11.

Finally, glucocorticoids are permissive for a number of metabolic reactions. These include the calorigenic effects of glucagon and catecholamines, secretion of aldolase and the dietary induction of pyruvate kinase (reviewed in (Lemaigre and Rousseau,

1994)). Glucocorticoids are also required for catecholamines to exert their lipolytic effects and produce pressor responses and bronchodilation (Ganong, 1989).

1.9.5 Glucocorticoids and the immune system

GR are found at varying levels throughout the immune system. The highest levels of GR are found in immature thymocytes, followed by mature splenocytes and peripheral lymphocytes with the lowest levels in neutrophils (Lowy, 1989) (Miller et al., 1998). GR in lymphocytes increases if the cells are activated e.g. by a mitogen or immunogenic stimulation (Munck et al., 1979) and GR is down-regulated as T cells mature (Miller et al., 1998), which may be related to the role of GR in thymocyte selection (section 1.9.5.2).

MR are not detectable in the thymus (Lowy, 1989; Reul et al., 1989) but are found at low levels in the spleen (Spencer et al., 1993).

1.9.5.1 Effects on inflammatory and immune responses

Both systemic and local release of glucocorticoids can regulate inflammatory and immune responses (reviewed in (McEwen et al., 1997; Sternberg, 2001)). At pharmacological doses glucocorticoids are generally immunosuppressive and affect activation, proliferation, differentiation and distribution of lymphocytes (reviewed in (McEwen et al., 1997; Sternberg, 2001)) and alter the function of other immune cells such as monocytes (reviewed in (Cupps and Fauci, 1982; McEwen et al., 1997)). If inflammatory responses are dominant in the pathogenesis of a disease then high levels of glucocorticoids may reduce its severity, whereas if pathogen effects dominate glucocorticoids will increase morbidity (reviewed in (Sternberg, 2001)).

Current evidence suggests that physiological concentrations of glucocorticoids are immunomodulatory, shifting cytokine production from a pro-inflammatory TH1 pattern of mainly IL-4 and interferon-γ production to an anti-inflammatory TH2 pattern of mainly IL-4 and IL-10 production (reviewed in (Elenkov and Chrousos, 1999)). Conversely, stress levels of glucocorticoids have distinct effects on individual cytokines (DeRijk et al., 1997) and HPA axis overactivity can increase susceptibility to infectious disease (Brown et al., 1993; Glaser and Kiecolt, 1998;

Hermann et al., 1993). The effects of glucocorticoids on cytokine expression may be modulated by activated GR interfering with the function of other transcription factors such as activator protein 1 (AP-1) and nuclear factor κB (NF-κB), which activate genes coding for many cytokines, cytokine receptors, chemotactic proteins and adhesion molecules (section 1.11.2, reviewed in (De Bosscher et al., 2000; McEwen et al., 1997)). Evidence from $GR^{dim/dim}$ mice suggests that this antagonism may be independent of DNA binding *in vivo* (Reichardt et al., 2000a).

The time of stress relative to exposure to immune stimulation and the type of stimulus are also important. For example, acute stress early in exposure actually increases the immune response in contact dermatitis (Dhabhar et al., 2000). Interestingly, low levels of glucocorticoids due to HPA axis underactivity can increase susceptibility to autoimmune and inflammatory disease. Lewis rats, which have a hyporesponsive HPA axis (Oitzl et al., 1995), develop arthritis in response to injected streptococcal antigen while Fischer rats with normal HPA axis responses do not (Sternberg et al., 1989). Lewis rats have decreased pituitary GR compared to Wistar rats (Oitzl et al., 1995) and have larger thymi than Fischer rats (Sternberg et al., 1989). It is tempting to speculate that the differences in immune responses between these animals is at least partly due to differences in GR levels in their immune cells, but these have not yet been measured.

As well as modulating immune function by influencing cytokine production, glucocorticoids may cause apoptosis of immune system cells. Lymphoid cells, especially $CD4^+CD8^+$ thymocytes, are sensitive to glucocorticoid-mediated apoptosis, a process that, as discussed below, is of particular relevance in T cell selection (reviewed in (Ashwell et al., 2000)). Although glucocorticoids are known to induce an endonuclease-like activity causing rapid cleavage of lymphocyte DNA (Compton and Cidlowski, 1986) and may stimulate intracellular free fatty acid release causing nuclear membrane damage (Turnell and Burton, 1975), the exact mechanism of glucocorticoid-induced apoptosis is unclear. However, most evidence suggests that they act via GR. GR^{AS} mice show an approximately 50% reduction in thymocyte GR expression and thymocytes from these animals are unresponsive to

glucocorticoids (Morale et al., 1995). Thymocytes from $GR^{dim/dim}$ mice were refractory to glucocorticoid-mediated apoptosis (Reichardt et al., 1998), as were those from $GR^{AS(Thy)}$ mice (King et al., 1995) whereas mice expressing a GR sense transgene in the thymus showed a twofold increase in thymocyte apoptosis compared to controls (Pazirandeh et al., 2002). Also, thymocytes from YGR mice express 20-24% more GR mRNA than controls and show greatly increased sensitivity to glucocorticoid-mediated apoptosis (Reichardt et al., 2000b).

Glucocorticoid-mediated apoptosis is ATP-dependent and blocked by protein-synthesis inhibitors, suggesting that they induce the expression of one or more genes causing cell death (reviewed in (Ashwell et al., 2000)). Bcl-2 (an inhibitor of mitochondrial-dependent cell death) inhibits glucocorticoid-mediated apoptosis of thymocytes and a lack of Bcl-2 expression appears to account for the glucocorticoid-sensitivity of $CD4^+CD8^+$ thymocytes (reviewed in (Ashwell et al., 2000)). Also, recent work using DNA chip technology demonstrated that glucocorticoid induction of GR expression is a primary phenomenon in glucocorticoid-induced T cell apoptosis and suggested that consequent GR-mediated repression of various metabolic pathways might lead to cell cycle arrest and cell death (Tonko et al., 2001).

The immune system also influences the HPA axis. Inflammatory mediators can affect central nervous system activity (reviewed in (Sternberg, 2001)). Cytokines can actively or passively cross the blood-brain barrier and stimulate neurons directly: intracerebroventricular injection of tumour necrosis factor caused a 51% decrease in hippocampal GR binding capacity (Betancur et al., 1995). They may also act indirectly by activating second messenger systems e.g. cyclooxygenase or by stimulating signaling in peripheral nerves e.g. the vagus (Bluthe et al., 1994; Watkins et al., 1994). These inputs collectively increase HPA axis activity and hence glucocorticoid levels, possibly reducing immune system activity and preventing the immune system from overreacting and causing autoimmunity (Munck et al., 1984).

1.9.5.2 Glucocorticoids and the thymus

In rats, T cell precursors appear in the thymus from days 13-14 of gestation, with $CD4^+CD8^+$ cells appearing from day 18 and mature thymocytes from day 20 (Vicente et al., 1998). At least 4 T cell receptor (TCR) dependent selection steps occur in the thymus (reviewed in (Godfrey et al., 2000) and (Ashwell et al., 2000)):

(a) T cell receptor-β mediated selection of cells undergoing the transition between $CD4^-CD8^-$ (double-negative, DN) and $CD4^+CD8^+$ (double positive or DP) states.

(b) "Death by neglect" of thymocytes bearing TCRs with subthreshold affinity for self-antigen/MHC (major histocompatibility factor) complexes.

(c) Positive selection leading to rescue from the default death pathway of DP cells bearing TCRs with intermediate affinity for self-antigen/MHC complexes, leading to their maturation into single-positive cells and migration to the periphery.

(d) Negative selection and elimination of thymocytes bearing TCRs with excessive affinity for self-antigen/MHC complexes, promoting self-tolerance.

The thymic endocrine environment is complex and is influenced by various extrinsic (e.g. prolactin, thyroxine, androgens and oestrogens) and putative intrinsic (thymosin α, thymulin and thymopoeitin) hormones as well as locally produced cytokines (reviewed in (Hadden, 1992)). Many pieces of evidence suggest that GR is crucial for thymocyte selection, although this area is highly controversial and the subject of much current debate.

GR levels change during thymocyte selection, since mature T cells possess less GR than immature thymocytes (Ranelletti et al., 1987) and different T cell subpopulation possess different levels of GR (Wiegers et al., 2001).

Glucocorticoids seem to be essential for thymocyte survival through selection. GR^{AS} mice show a partial blockade of T cell differentiation in the embryo and altered

thymic stromal architecture in the adult (Sacedon et al., 1999b). $GR^{AS(Thy)}$ mice showed a thymus-specific 43% reduction in GR expression and impaired glucocorticoid-mediated gene transcription (King et al., 1995). These mice showed reduced thymocyte numbers principally due to a reduction in $CD4^+CD8^+$ cells (King et al., 1995). They also had increased frequency of $CD4^-CD8^-$ thymocytes compared to controls (King et al., 1995). The reduction in absolute numbers of $CD4^+CD8^+$ is greater than that of $CD4^-CD8^+/CD4^+CD8^-$ thymocytes, with numbers of $CD4^-CD8^-$ cells unchanged (King et al., 1995). These mice also show increased spontaneous apoptosis and increased sensitivity to TCR-mediated deletion of $CD4^+CD8^+$ cells (King et al., 1995). Thymic epithelial cells express steroidogenic enzymes (Lechner et al., 2000; Pazirandeh et al., 1999; Vacchio et al., 1994) and produce glucocorticoids in cell culture (Pazirandeh et al., 1999). Inhibition of glucocorticoid synthesis by metyrapone in foetal thymus organ culture from non-transgenic mice blocked the transition of thymocytes from DN to DP, an effect that was reversed by corticosterone administration (King et al., 1995). Metyrapone also increased apoptosis in DP cells with transgenic TCRs which recognized self-antigen/MHC complexes (Vacchio and Ashwell, 1997). Furthermore, inhibition of thymic corticosteroid production enhanced TCR-mediated antigen-specific deletion of immature thymocytes (Vacchio et al., 1994). These data suggest that glucocorticoids play a role in the survival of thymocytes during the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ cells.

However, glucocorticoids may cause cell death during thymocyte selection. Glucocorticoids increased thymocyte apoptosis after injection of a suboptimal dose of anti-CD3 (Jondal et al., 1993). Glucocorticoids secreted in the thymus induce apoptosis in cocultured T cells, an effect partially blocked by the glucocorticoid synthesis inhibitor metyrapone and the GR antagonist RU486 (Miller et al., 1990) and may account for persistence of T cell apoptosis after maternal adrenalectomy (Sacedon et al., 1999a). Furthermore, *in vivo* administration of the GR antagonist RU486 blocked DP thymocyte apoptosis induced by injection of anti-CD3 antibody (Jondal et al., 1993) and inhibited TCR-mediated thymocyte apoptosis (Xue et al., 1996). RU486 also inhibited TCR-mediated thymocyte apoptosis in TCR transgenic

thymi *in vivo* and *in vitro* (Xue et al., 1996). However, RU486 has also been shown to increase TCR-mediated apoptosis of DP cells in FTOC (Vacchio et al., 1994): these different results may be due to variations in the dose of RU486 used (reviewed in (Godfrey et al., 2000)).

To explain these apparently conflicting roles for glucocorticoids in selection, Ashwell *et al* have proposed the "mutual antagonism" model whereby the balance between glucocorticoid and TCR signaling controls selection (Ashwell et al., 2000). The model proposes that DP thymocytes with subthreshold avidity for self-antigen/MHC undergo death by neglect at least partially due to glucocorticoid-mediated apoptosis, while encounter of self-antigen/MHC by thymocytes with intermediate-avidity TCRs leads to signaling that would otherwise lead to apoptosis, but due to glucocorticoid antagonism the cells survive. Also, they propose that signals leading to apoptosis in thymocytes bearing TCRs with high avidity for self are too strong to be overcome by ambient glucocorticoids and these cells are deleted by negative selection. In support of this hypothesis, it is known that TCR signaling can antagonize glucocorticoid-mediated apoptosis (Iwata et al., 1991; Zacharchuk et al., 1990) and the TCR and GR signaling pathways are linked via the Ras and MEK signaling components (Jamieson and Yamamoto, 2000).

However, some evidence suggests that glucocorticoids may not be involved in T cell selection. Thymocytes from $GR^{dim/dim}$ mice show resistance to glucocorticoid-mediated apoptosis, but these animals show no difference in their relative abundance of T lymphocyte subpopulations from control animals (Reichardt et al., 1998). However, the GR in these animals might still be influencing selection by GR monomers interacting with other transcription factors (as discussed in section 1.9.5). Also, $GR^{hypo/hypo}$ mice (Cole et al., 1995a) have T cells unresponsive to dexamethasone-mediated apoptosis, but normal positive and negative selection processes occur (Purton et al., 2000). However, since the majority of mice with this mutation die at birth due to lung atelectasis (Cole et al., 1995a) all these studies were carried out in organ culture and therefore do not necessarily represent the situation *in vivo*. Also, these mice do express an N-terminus truncated form of GR which may

still have DNA-binding ability (Cole et al., 2001; Tronche et al., 1998), which may retain sufficient function to allow normal thymocyte selection to proceed.

Despite the evidence presented above, on balance I believe that the evidence favours a central role for glucocorticoids in thymocyte development. Nevertheless, further work is required to clarify the exact role of glucocorticoids in the thymus.

1.10 Gene transcription in eukaryotes

Gene expression may be controlled at various stages including transcription, RNA processing, RNA transport, mRNA degradation, translation and post-translationally. In most genes transcriptional controls are paramount: these are mediated by the interactions of gene regulatory proteins with the control region of the gene, changes in the chromatin structure of the gene and changes in the methylation status of the gene (Alberts B et al., 1994).

1.10.1 Control of transcription by gene regulatory proteins

The control region of a eukaryotic gene consists of the DNA sequences required to initiate transcription and regulate the rate at which this initiation occurs, i.e. the promoter and various short (<20 nucleotides) regulatory sequences of varying complexity to which gene regulatory proteins (specific transcription factors) bind (Figure 1.7).

The majority of promoters have the same core sequences, the TATA box and the initiator, although promoter structure varies widely (reviewed in (Zawel and Reinberg, 1995)). Various initiator families exist (reviewed in (Weis and Reinberg, 1992)), with an underlying sequence similarity (Javahery et al., 1994). The initiator can initiate transcription in TATA-less promoters (reviewed in (Weis and Reinberg, 1992; Zawel and Reinberg, 1995)), as discussed below, and indeed may be the dominant influence on transcriptional machinery assembly (reviewed in (Zawel and Reinberg, 1995)).

The transcriptional machinery consists of RNA polymerase II and the general transcription factors (Alberts B et al., 1994). Transcription is initiated by TFIID

binding to the TATA box via its TATA binding protein subunit (TBP), which is also required for transcription initiation in TATA-less promoters. Subsequently the rest of the transcriptional machinery assembles at the transcription start site (reviewed in (Pugh, 1996; Zawel and Reinberg, 1995)), probably as a PolII holoenzyme (reviewed in (Maldonado and Reinberg, 1995; Pugh, 1996)) and transcription begins.

Each transcription factor or family of transcription factors (Faisst and Meyer, 1992) can bind to several of the hundreds of different regulatory sequences (Kel et al., 1995) via specific protein-DNA interactions (Alberts B et al., 1994). These occur through several protein motifs, including helix-turn-helix, β-sheet, zinc finger, leucine zipper and helix-loop-helix motifs (reviewed in (Garvie and Wolberger, 2001)).

Different combinations of transcription factors (some of which, activators, promote initiation while others, repressors, inhibit initiation) are present in different cell types (Alberts B et al., 1994). This allows cell and tissue-specific regulation of gene expression. Most transcription factors work as part of a complex of several proteins whose activator or repressor function depends on the final assembly of all the individual components, including cofactors (Alberts B et al., 1994). Two types of cofactor exist: coactivators that enhance the response of the transcriptional machinery to activators and corepressors that repress basal transcription (reviewed in (Zawel and Reinberg, 1995)).

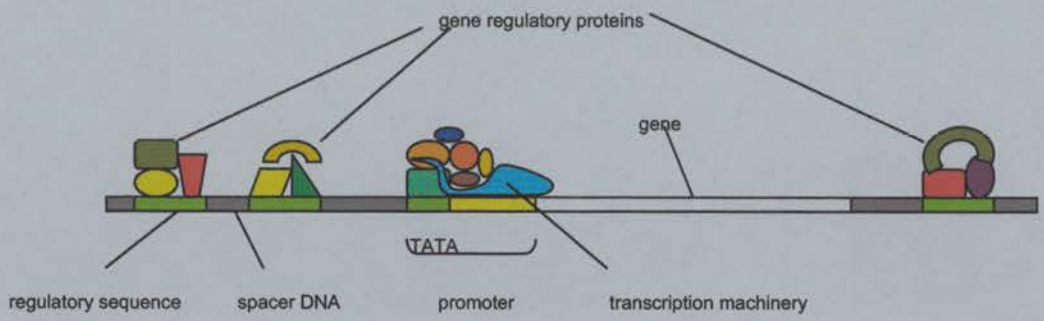


Figure 1.7: the gene control region of a typical eukaryotic gene. Adapted from (Alberts B et al., 1994).

1.10.2 Control of transcription by alternate promoter usage

Alternate promoter usage has been observed for many genes. The general consensus is that by transcribing one gene from multiple promoters the organism gains additional flexibility in control of expression of the gene. Various patterns of alternate promoter usage exist of varying complexity (reviewed in (Ayoubi and VanDeVen, 1996)).

The simplest model has two tandemly arranged promoters closely positioned within the same exon (coding or noncoding) e.g. in the MYC oncogene (Marcu et al., 1992). Alternatively, multiple promoter usage may lead to noncoding alternate first exons spliced onto a common exon 2, as found in the MR (Vazquez et al., 1998; Zennaro et al., 1995; Zennaro et al., 1996), ER (Flouriot et al., 1998), GR (section 1.6), α-amylase (Schibler et al., 1983) and HOX5.1 (Cianetti et al., 1990) genes.

In the case of the mineralocorticoid receptor, these alternate promoters show tissue and region-specific differences in their relative expression. In the hippocampus, the MR mRNA variant associated with activity of the α promoter is most abundant in CA2 and dentate gyrus, while the ß and γ variants are evenly distributed through CA1-4 (Kwak et al., 1993). Also, these variants are differentially regulated by glucocorticoids, with the α variant specifically upregulated by adrenalectomy (Kwak et al., 1993). The activity of these promoters also varies through neuronal development, with activity of the ß promoter associated with synaptogenesis and activity of the γ promoter associated with cell birth and axonal sprouting (Vazquez et al., 1998).

Likewise, a total of six variant human ERα mRNAs with different 5' untranslated regions are generated by alternate promoter usage (Flouriot et al., 1998). The promoters show differential activity; for example, the A and B promoters are active in the forebrain but the C promoter is not (Osterlund et al., 2000). Six similar ERα variant mRNAs are found in the mouse, again generated by alternate promoter usage and splicing (Kos et al., 2000). All these variants show a tissue and sex-specific

pattern of expression, with the C and F variants predominantly expressed and the H variant restricted to liver, although female mice produce an approximately five-fold higher level of this transcript than males (Kos et al., 2000).

Several variations of noncoding alternate first exons spliced onto a common exon 2 occur depending on the arrangement of the promoters and translation start sites. The most complex models involve multiple promoters which lead to the formation of mRNAs encoding different protein isoforms (reviewed in (Ayoubi and VanDeVen, 1996)). The distance between these promoters may be large e.g. in the dystrophin gene, where the alternate promoters are separated by more than 500kb (Nishio et al., 1994). In the progesterone receptor gene, whose overlapping leader exons contain multiple promoters, transcription initiation from the upstream promoter leads to mRNA products encoding a protein with an amino-terminal extension (Kastner et al., 1990). Proteins with an amino-terminal extension can also be produced from use of upstream promoters associated with non-overlapping first exons followed by a splicing event e.g. in the human porphobilinogen deaminase (PBGD) gene (Chretien et al., 1988), the glucokinase gene (Iynedjian, 1993), the catecholamine-O-methyltransferase gene (Tenhunen et al., 1993) and the c-myb proto-oncogene (Jacobs et al., 1994). These different isoforms are sometimes involved in gene autoregulation e.g. in the CREM inducible cAMP early repressor isoform (Molina et al., 1993). Interestingly, transcription initiation at the human GR gene promoter associated with exon 1A has been suggested to encode membrane-associated GR (mGR) or direct GR to the plasma membrane (Gametchu et al., 1999), although it is unclear how this might occur since the in-frame stop codon at the start of exon 2 should mean that transcripts containing different exons 1 produce identical GR proteins.

A single promoter is unlikely to contain all the *cis*-acting control elements needed for tissue or development-specific expression of a gene and for its response to hormones or other stimuli. Conversely, use of multiple promoters can confer additional flexibility in the control of gene expression and is likely to have several functions (reviewed in (Ayoubi and VanDeVen, 1996)). Multiple promoter usage can allow tissue-specific regulation of gene expression, e.g. in the PBGD gene which possesses

both an upstream, ubiquitously-active "housekeeping" promoter and a downstream tissue-specific promoter active only in erythroid cells (Chretien et al., 1988). Alternate promoters of different strengths may be active in different tissues e.g. in the α-amylase gene a weak downstream promoter is active in liver and a strong upstream promoter is active in the parotid gland, giving a 100-fold difference in expression between these tissues (Schibler et al., 1983). Tissue-specificity may also be conferred by different responsiveness of promoters to transcription factors e.g. in the glucokinase gene the downstream promoter is regulated by insulin and glucagon but the upstream is not (Iynedjian, 1993) and the MR and ER genes (see above).

Alternate promoter usage also enables temporal and spatial regulation of gene expression during development e.g. in the IGF-II gene, where 3 promoters (P2, P3 and P4) are active in the liver during foetal development but shut off at birth, at which time the P1 promoter is activated (van Dijk et al., 1992).

Production of alternate mRNAs with different stabilities or translational efficiencies by alternate promoter usage allows post-transcriptional regulation of gene expression, e.g. in the HOX-5.1 gene where 2 promoters give rise to mRNA isoforms with different 5'-untranslated regions, tissue and subcellular distributions, induction by retinoic acid and stability (Cianetti et al., 1990). Some long GC-rich leader exons produced by alternate promoters are inefficiently translated and many leader exons contain upstream AUGs which are involved in translation control (Kozak, 1991). These AUGs are uncommon except in oncogenes (Kozak, 1987), suggesting they may function to attenuate inappropriate translation (reviewed in (Ayoubi and VanDeVen, 1996)).

1.10.3 Control of transcription by modifications of chromatin

The degree of compaction of chromatin can affect transcription. Nucleosomes repress transcription initiation at promoters contained within them (reviewed in (Wu and Grunstein, 2000)) and higher-order DNA packing (e.g. heterochromatin) renders the DNA inaccessible for transcription (Alberts B et al., 1994).

The mechanisms of chromatin decondensation to allow transcription are poorly understood. However, activator protein binding is thought to recruit several classes of chromatin-modifying proteins (reviewed in (Sterner and Berger, 2000; Wu and Grunstein, 2000)):

(a) Switch/sucrose non fermentable (SWI/SNF) and related proteins reposition nucleosomes in *cis*, may transfer histone octamers in *trans* to other DNA molecules, may also destablise the nucleosome by removing H2A-H2B dimers and have been shown to create superhelical torsion in DNA *in vitro*.

(b) Histone acetyltransferases (HATs) including those of the GNAT, MYST and P300/CBP families, some nuclear receptor cofactors e.g. SRC-1, ACTR and TIF2 and possibly one subunit of TFIID all modify the acetylation status of histones. Their importance is illustrated by HAT mutations being associated with dysregulation of gene expression in certain tumours and developmental disorders (reviewed in (Marmorstein and Roth, 2001)). Acetylation reduces the strength of binding of histones to DNA and each other, thus releasing DNA from the nucleosome and possibly disrupting higher-order nucleosomal structures. Histone deacetylation strengthens these interactions and represses transcription by a poorly defined mechanism. Certain corepressors stabilise chromatin by targeting histone deacetylases to specific sections of DNA (reviewed in (Collingwood et al., 1999)). Genome-wide acetylation and deacetylation of histones occurs, promoting a rapid turnover of acetyl groups and preventing targeted acetylation or deacetylation from permanently affecting chromatin structure.

Binding of some activators is thought to have the net effect of "opening" the chromatin structure around the promoter allowing access and assembly of the transcriptional machinery, while others are thought to bind to DNA once the chromatin has opened (Alberts B et al., 1994). Repressor binding is thought to have the opposite effect (Alberts B et al., 1994). Other covalent modifications of histones e.g. phosphorylation, methylation and ubiquitination occur: their effect on chromatin structure is poorly understood. Methylation of histones may be involved in

epigenetic silencing of gene expression and genomic imprinting (reviewed in (Rice and Allis, 2001)).

1.10.4 DNA methylation and transcription

Methylation of DNA occurs at the sequence 5'-CpG-3' (or rarely at the sequence 5'-CpNpG-3') (reviewed in (Newell-Price et al., 2000)). CpG islands are 500-2000bp areas of genomic DNA that, unlike bulk DNA, are not deficient in CpG (Bird et al., 1985). They often lie in the promoter region of genes, but are also found within the structural gene (reviewed in (Jones, 1999)). Methylation of the cytosine residue is performed by one of 3 DNA methyltransferases (reviewed in (Newell-Price et al., 2000)).

The pattern of methylation in the genome is established during embryogenesis. In the preimplantation embryo the methylation pattern of the gamete is erased, then after implantation *de novo* methylation occurs at the vast majority of CpGs (reviewed in (Jaenisch and Jahner, 1984)). The approximately 60% of promoters that lie within CpG islands escape *de novo* methylation (reviewed in (Newell-Price et al., 2000)). This may be due to binding of the transcription factor SP1, which can bind to methylated or unmethylated DNA (Silke et al., 1995), at sites in or near the CpG island. SP1 elements have been shown to protect a CpG island in the adenine phosphoribosyltransferase (APRT) gene from *de novo* methylation (Brandeis et al., 1994) and deletion or mutagenesis of SP1 binding sites causes *de novo* methylation of the APRT gene CpG island in a transgenic mouse assay (Macleod et al., 1994). An embryo-specific element in the sequence of the APRT gene CpG island can protect itself from *de novo* methylation and reduce methylation of flanking sequences in transgenic mice (Siegfried et al., 1999), suggesting that other factors apart from SP1 may help establish methylation patterns in the embryo.

Methylation is involved in gene silencing in several physiological processes e.g. X chromosome inactivation, parental imprinting and developmental regulation of gene expression (reviewed in (Newell-Price et al., 2000)). Defects in developmental DNA methylation are implicated in the pathogenesis of several diseases including the Rett and fragile X syndromes (reviewed in (Robertson and Wolffe, 2000)).

Methylation-induced repression of transcription may occur by several mechanisms (reviewed in (Jones, 1999; Newell-Price et al., 2000)):

(a) The methyl groups protrude into the major groove of the DNA helix, possibly interfering with transcription factor binding

(b) Methylation recruits CpG binding proteins that mediate repression, such as MeCp2

(c) Methylation recruits histone deacetylases and corepressors, causing the formation of condensed, transcriptionally inactive chromatin

(d) Methylation of a promoter may directly interfere with the transcriptional machinery

Although important in developmental regulation of gene expression, methylation of promoters in CpG islands may be involved in dynamic regulation of gene expression. The POMC gene promoter is in a CpG island and is methylated in tissues that do not express the gene, while methylation *in vitro* silences POMC gene expression (reviewed in (Newell-Price et al., 2000)). Also, methylation of the androgen receptor promoter CpG island leads to loss of androgen receptor expression in prostatic tumour cells (Jarrard et al., 1998). These data suggest that methylation may regulate the tissue-specific expression of genes whose promoter lies in a CpG island.

1.11 Transcriptional regulation by the glucocorticoid receptor

1.11.1 Transcriptional activation

Recent studies utilizing fusions of GR with green fluorescent protein have shown that GR interacts dynamically with regulatory elements (a "hit and run" model) rather than binding to them in a static manner (McNally et al., 2000). When associated with DNA at a positive GRE (section 1.5.1), GR appears to activate transcription in two ways.

GR binding causes both chromatin rearrangement and appearance of DNase hypersensitive sites in the rat tyrosine aminotransferase enhancer (Reik et al., 1991)

and nucleosome phasing in studies using the mouse mammary tumour virus long terminal repeat (MMTV LTR) (reviewed in (Beato, 1989)). This suggested that GR might increase the rate of transcription by remodeling chromatin and improving access of the transcriptional machinery and other transcription factors to the promoter (section 1.10.3). Although the mechanism by which GR binding alters chromatin structure is not fully understood, the extensive recent studies of GR-mediated chromatin remodeling on the MMTV LTR (e.g. (Fletcher et al., 2000), reviewed in (Deroo and Archer, 2001)) suggest that ligand-activated GR enters the nucleus, recruits various other chromatin-remodelling proteins such as those of the SWI/SNF family and delivers them to the promoter while transiently interacting with the GRE (reviewed in (Deroo and Archer, 2001)), also displacing the linker histone H1 (Bresnick et al., 1992). Subsequently the interaction of DNA with the core histones is altered, allowing the binding of other transcription factors such as NF-1 and Oct-1 (Archer et al., 1992; Truss et al., 1995), recruitment of the transcriptional machinery and initiation of transcription (reviewed in (Deroo and Archer, 2001)).

Binding of GR to the GRE also serves to locate the GR next to the promoter where it may increase transcription by interacting with the general transcription factors (section 1.10.1) to increase recruitment of the transcriptional machinery (reviewed in (Beato et al., 1996)). Evidence suggests that GR usually acts in combination with other transcription factors to cause transcriptional activation. GR seems to mediate the assembly of transcription factor complexes on the MMTV promoter (Cordingley et al., 1987) and various transcription factors including NF1, CP1, SP1 and OTF can interact synergistically with GR to stimulate transcription when their binding sites lie within 350bp of the GRE (Schule et al., 1988; Strahle et al., 1988). Also, GR interacts with HNF-5 on the rat tyrosine aminotransferase gene enhancer (Rigaud et al., 1991), regulates the binding of CEBPβ to the alpha-1-acid glycoprotein promoter in rat hepatoma cells (Savoldi et al., 1997) and interacts synergistically with STAT5 to induce transcription at the β-casein promoter (Stocklin et al., 1996). Furthermore, DNA binding of GR does not seem to be necessary for it to interact with other transcription factors. In GR dimerisation-deficient $GR^{dim/dim}$ mice glucocorticoid-induced genes e.g. PNMT and surfactant protein are still expressed, suggesting that

crosstalk of GR with other transcription factors may be stimulating their transcription (Reichardt et al., 1998).

GR can also facilitate transcription complex assembly by interacting with various coactivators or transcription intermediary factors (TIFs) e.g. DRIP150 and DRIP205 (Hittelman et al., 1999), SRC-1, GRIP1/TIF2, ARA70, Rap46 and RSP5 (reviewed in (Jenkins et al., 2001; Robyr et al., 2000)) and GRIP170, a protein which increases GR-mediated transcription in a cell-free system through complexing with GR (Eggert et al., 1995). These coactivators require the presence of a nuclear hormone receptor for their recruitment to the promoter, enhance the activation function of GR and interact with components of the transcription machinery. Additionally, SRC-1 and p/CAF possess histone acetyltransferase activity (reviewed in (Struhl, 1998)). As yet, the only corepressors shown to interact with GR are calreticulin and TSG101 (section 1.11.2).

GR activation can affect transcription by inducing changes in the methylation status of DNA (section 1.10.4). 3d glucocorticoid treatment results in demethylation of all 4 methyl CpGs within the rat TAT gene enhancer, allowing stronger and faster subsequent responses of the enhancer to glucocorticoids (Thomassin et al., 2001). The mechanism of GR-induced demethylation is unclear, but the presence of DNA strand breaks during the process may suggest an active mechanism involving base excision (Kress et al., 2001).

The DNA binding and transactivation activity of many transcription factors is affected by their phosphorylation status (reviewed in (Hunter and Karin, 1992)). Transcriptional activation by GR may be modulated by changes in receptor phosphorylation. All steroid hormone receptors are phosphorylated and undergo hormone-dependent hyperphosphorylation; in GR (typically for the receptor family) this occurs mostly in the transactivation regions of the N-terminal domain (reviewed in (Bodwell et al., 1998)). GR hyperphosphorylation decreases receptor half-life (Webster et al., 1997) but strongly enhances transcriptional activation, although this effect is highly promoter-specific (reviewed in (Bodwell et al., 1998)). For example, GR-mediated recruitment of HNF-3 to the rat tyrosine aminotransferase gene

enhancer is dependent on protein kinase A signaling and transcriptional activation by the GR ligand RU486 is enhanced by increased intracellular cyclic AMP or phosphatase inhibitor administration (reviewed in (Beato et al., 1996)). Also, phosphorylation enhances transrepression of the GR gene by GR (Webster et al., 1997). However, GR transactivation of the MMTV promoter in COS-1 cells was not affected by hyperphosphorylation (Webster et al., 1997). MR function is also affected by protein kinase A, probably by an indirect mechanism relieving the effect of an MR repressor (Massaad et al., 1999): a similar mechanism may apply to GR. Inhibition of tyrosine kinase activity using herbimycin A show that phosphorylation by tyrosine kinase is involved in maintenance of GR protein levels, probably by increasing receptor stability (Niimi et al., 1997).

1.11.2 Transcriptional repression

GR downregulates the expression of several genes, such as the POMC gene (section 1.9.2.2) and the genes encoding several cytokines (section 1.9.5.1). Several mechanisms of GR repression have been proposed.

GR may repress transcription by binding to negative GREs (nGREs). These were first discovered in the POMC, prolactin and α-subunit of glycoprotein promoters and are more variable in sequence than the positive GRE (reviewed in (Beato, 1989; Karin, 1998)). However, it appears that it is not the precise sequence of the DNA that makes a GRE negative but rather its particular location in the context of the regulatory region of the gene (Pearce and Yamamoto, 1993). This is exemplified by the α-subunit of glycoprotein gene, where the GR binds to a 52 nucleotide region that also contains elements essential for cAMP responsiveness and placental-specific expression of the gene and only causes transrepression in tissues where these elements are functioning as enhancers (Akerblom et al., 1988). When bound to the nGRE GR may interfere with the function of the transcriptional apparatus e.g. in the POMC gene where GR binds to the nGRE at -60 (Drouin et al., 1993). It has been suggested that GR binds as a trimer (Drouin et al., 1993) and prevents TFIID binding to the TATA box (Drouin et al., 1987). GR may also interfere with the binding of other transcription factors e.g. in the proliferin promoter and glutamine synthase

enhancer where it may interfere with AP-1 binding (reviewed in (Beato et al., 1996)), the CRH (Malkoski et al., 1997) and α-subunit of glycoprotein (Akerblom et al., 1988) promoters where it may interfere with the function of a cyclic AMP response element and the globin gene of mouse erythroleukaemia cells where it can block GATA-1 binding (reviewed in (Beato et al., 1996)).

GR may also repress transcription by direct interaction with other transcription factors in the nucleus, e.g. HSF1 (Wadekar et al., 2001), AP-1, NF-κB, GATA-1 and CREB (reviewed in (Beato et al., 1996; Reichardt and Schutz, 1998)). Studies showing that GR can inhibit AP-1 mediated induction of genes lacking GREs e.g. the collagenase gene (reviewed in (Beato et al., 1996)) and that an activation-deficient GR mutant with no dimerisation ability can repress AP-1 activity in cultured Jurkat cells (Heck et al., 1994; Helmberg et al., 1995) suggest that this interaction does not require DNA binding by GR. These interactions may involve masking of transactivation domains, post-translational modifications or conformational changes in the proteins (reviewed in (Beato et al., 1996; Reichardt and Schutz, 1998)) and are likely to be complex with crosstalk between transcription factor systems e.g. AP-1 signaling can lead to increased circulating levels of glucocorticoids and eventual down-modulation of AP-1 signaling in a negative feedback loop (reviewed in (Karin and Chang, 2001)). Also, the activity of GR may be repressed by protein-protein interactions. For example, calreticulin binds to the GR DBD and prevents its binding to GREs (Burns et al., 1994; Dedhar et al., 1994) and tumour susceptibility gene 101 (TSG101) represses the transactivation activity of the AF-1 domain of GR (Hittelman et al., 1999).

As well as interacting with them directly, GR may compete with other transcription factors e.g. NF-κB for binding to HAT coactivator proteins (section 1.10.3) such as CREB binding protein (CBP) or steroid receptor coactivator-1 (Sheppard et al., 1998). However, although there is evidence that coactivators may be limiting in the process of transactivation, this is not the case for GR-mediated transrepression in a model combining p65, CBP and GR (De Bosscher et al., 2000) so the physiological relevance of this model is uncertain.

Finally, GR may interfere with the action of other transcription factors by increasing the synthesis of inhibitor proteins which bind them and maintain them in an inactive state. An example of this is IκB-α, which binds NF-κB in an inactive complex in the cytoplasm (Auphan et al., 1995; Scheinman et al., 1995). This induction of IκB-α is not seen in all cell types, however, and the mechanism by which it might occur is unclear since the IκB-α promoter does not contain a GRE (De Bosscher et al., 2000).

These various models are not mutually exclusive and it seems likely that GR transrepression is due to a combination of the above effects.

1.12 Aims

This thesis examines the tissue-specific expression and glucocorticoid autoregulation of variant exons 1 of the GR gene. GR is widely expressed in the body and has important physiological effects. In particular, GR is highly expressed in the hippocampus, where changes in GR levels alter HPA axis regulation and hence affect all physiological processes that are mediated by glucocorticoids. GR is also highly expressed in the thymus, where it is important in the development and function of the immune system and in the liver, where it modulates the effects of glucocorticoids on hepatic metabolism.

Although glucocorticoid autoregulation of GR levels is well-recognised and is almost certainly fundamentally important in both dynamic and programmed changes in GR expression, surprisingly little is known about the mechanisms of transcriptional regulation of the GR gene. The GR gene is large and complex; multiple 5' untranslated exons 1 have been identified in the rat, mouse and human GR genes. These variant exons 1 are thought to arise from transcription initiation at alternate promoters. Furthermore, both tissue-specific control of GR levels and perinatal programming of GR expression may involve changes in alternate promoter usage (e.g. prenatal dexamethasone exposure has been shown to affect the expression of exons 1_7 and 1_{10} of the rat GR gene).

The work described in this thesis was designed to further investigate whether tissue-specific changes in alternate promoter usage have an important role in transcriptional

control of the GR gene. In order to do this, the work planned to test 3 main hypotheses. The first was that there were widespread tissue and region-specific differences in the pattern of expression of variant exons 1 of the GR gene (and hence possibly alternate promoter usage), with the expression of certain exons 1 being restricted to specific tissues. The second hypothesis was that changes in the pattern of variant exon 1 expression would be associated with tissue and region-specific differences in GR expression. To investigate these hypotheses, the pattern of variant exon 1 expression and the distribution of variant transcripts relative to that of the total pool of GR mRNA were characterized in a range of tissues.

The third hypothesis arose from the evidence suggesting a role for alternate promoter usage in perinatal programming of GR levels. It was proposed that changes in alternate promoter usage were the mechanism by which glucocorticoids autoregulate GR levels in adult animals; specifically, that glucocorticoid-induced changes in the activity of several tissue-specific promoters (e.g. those associated with exons 1_1 and 1_7) but not those thought to be ubiquitously active (e.g the promoter associated with exon 1_{10}) might be the mechanism of autoregulation. To investigate this, this work studied the effects of glucocorticoid manipulations on expression of total GR mRNA and variant exons 1 in hippocampus, liver and thymus, where glucocorticoids are known to have important physiological effects.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals were obtained from one of the following two suppliers unless listed specifically below:

BDH Chemicals Ltd, Magna Park, Lutterworth, Leicestershire, LE17 4XN.

Sigma Chemicals Ltd, Fancy Road, Poole, Dorset, BH17 7NH.

Chemical	**Supplier**
Nucleotide triphosphates Scintillation proximity assay reagent	Amersham Pharmacia Biotech UK Ltd, Little Chalfont, Buckinghamshire, HP7 9NA.
Bactotryptone Yeast extract Agar	Becton Dickinson, Between Towns Road, Cowley, Oxford, OX4 3LY.
Kodak D19 developer Amfix High Speed Fixer	H.A.West Ltd, 41, Watson Crescent, Edinburgh, EH11 1ES.
Ethanol	Hayman Ltd, 70, Eastways Industrial Park, Witham, Essex, CM8 3YE.
Agarose/low melting point agarose Caesium chloride	Life Technologies Ltd, 3, Fountain Drive, Paisley, PA3 9RF.

2.1.2 Radiochemicals

All radiochemicals were supplied by Amersham Pharmacia Biotech UK Ltd.

Compound	Specific activity
[α^{32}P]-dCTP	110 TBq/mmol
[α^{32}P]-GTP	110 TBq/mmol
[α^{33}P]-dNTPs	16 MBq/ml
[α^{35}S]-UTP	30 TBq/mmol

2.1.3 Enzymes

Deoxyribonuclease I, Proteinase K, RNase A and RNase T1 were supplied by Roche Diagnostics Ltd, Bell Lane, Lewe, East Sussex, BN7 1LG. All other enzymes were supplied by Promega UK Ltd, Delta House, Chilworth Research Centre, Southampton, SO1 7NS.

2.1.4 Miscellaneous

Item	Supplier
Sephadex G-50 grade ("Nick") columns	Amersham Pharmacia Biotech UK Ltd
Hybspeed RPA Kit II Century RNA Marker Set	AMS Biotechnology, Milton Park, Abingdon, Oxfordshire.
Cryo-M-Bed embedding compound	Bright Instrument Co. Ltd, St Margaret's Way, Huntingdon, PE18 6EB.
Kodak autoradiographic film	H.A.West Ltd
Hybaid Recovery DNA Purification Kit II	Hybaid Ltd, Action Court, Ashford Road, Ashford, Middlesex, TW15 1XB.

Item	Supplier
DNA size markers (1Kb DNA ladder)	Life Technologies Ltd
TRIzol reagent	
Fluothane	Merial Animal Health Ltd, Sandringham house, Harlow Business Park, Harlow.
Reverse Transcription System	Promega Ltd
Vetergesic	Reckitt & Colman Products Ltd, Dansom Lane, Hull, HU8 7DS.

2.1.5 Solutions and buffers

2.1.5.1 General

Deionised water was used to make all solutions unless otherwise stated or if solutions were for RNA work, in which case ultrapure water was used. Solutions marked [1] were not autoclaved before use: all others were. Solutions marked [2] were treated with diethylpyrocarbonate (DEPC) (see section 2.1.5.1, DEPC water) before autoclaving if they were to be used for RNA work.

Solution	Composition
Alkaline SDS[1]	0.2M NaOH, 1% SDS
Borate buffer	0.13M boric acid, 46mM NaCl and 0.5% (w/v) bovine serum albumin. pH adjusted to 7.4 with conc. HCl.
"Box buffer" for *in situ* mRNA hybridization	20ml 20 x SSC, 50ml deionised formamide (not molecular biology grade), volume adjusted to 100ml with DEPC water.

Solution	Composition
Buffer I for nuclei preparation	0.32M sucrose, 0.1mM EDTA, 10mM Tris-HCl pH 8.0 and 1mM EGTA. Just before use, 0.1% Triton X-100, 1mM spermidine, 1.0mM DTT and 0.1mM PMSF were added.
Buffer II for nuclei preparation	2.0M sucrose, 5.0mM Mg acetate, 0.1mM EDTA and 10mM Tris-HCl pH 8.0. Just before use 1.0mM DTT and 0.1mM PMSF were added.
Deionised formamide	150ml formamide was mixed with 15g Amberlite mixed bed ion-exchange resin for 1hr at room temperature then filtered twice through Whatman No. 1 filter paper and stored at -20°C (for molecular biology grade) or room temperature (for non molecular biology grade).
DEPC water	0.5ml DEPC in 500ml ultrapure water. Leave for 1-24 hours, autoclave.
DNA loading buffer[1]	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol.
0.5M EDTA pH 8.0[2]	186.1g $Na_2EDTA.2H_2O$ was dissolved in 800ml water, pH was adjusted to 8.0 with NaOH and volume was adjusted to 1L.
50%, 70% and 90% ethanol in ammonium acetate[1]	11.55g of ammonium acetate was mixed with 250, 350 or 450ml of ethanol as

Solution	Composition
	appropriate and the volume was adjusted to 500ml with water.
Formamide loading buffer[1]	34.03% (w/v) bromophenol blue, 0.03% (w/v) xylene cyanol, 20mM EDTA in deionised formamide.
Glycerol tolerant buffer (20x)	216g Trizma base, 72g taurine and 4g $Na_2EDTA.2H_2O$ were dissolved to a final volume of 1L.
GTE	50mM glucose, 25mM Tris-HCl ph 8.0, 10mM EDTA
Hybridization buffer for *in situ* mRNA hybridisation (2x)[1]	1.2M NaCl, 20mM Tris-HCl pH 7.5, 2 x Denhardt's solution, 2mM EDTA, 0.2mg/ml sonicated single-stranded salmon testes DNA, 0.2mg/ml yeast tRNA, 20% dextran sulphate.
MEA buffer (10x)	200mM MOPS acid, 50mM sodium acetate and 10mM EDTA with pH adjusted to 7.2
Northern blot hybridisation buffer	0.13M NaH_2PO_4, 0.4M Na_2HPO_4, 3.3mM EDTA, 6.6% SDS and 10μg/ml sonicated single-stranded salmon testes DNA (denatured for 2min at 100°C before adding to the buffer).
4% paraformaldehyde in 0.1M phosphate buffer[1]	Dissolve 20mM NaH_2PO_4 and 80mM Na_2HPO_4 in 1L DEPC water, heat to 80°C then add 40g paraformaldehyde.

Solution	Composition
	Stir for 1 hour, cool, then store at 4°C.
PBS (10x) [2]	80g NaCl, 2g KCl, 2g KH_2PO_4, 29g $Na_2HPO_4.2H_2O$ were dissolved in 800ml water. PH was adjusted to 7.4 and the volume adjusted to 1L.
5M potassium acetate (5M with respect to acetate, 3M with respect to potassium)	245.6g potassium acetate was dissolved in 300ml water. Volume was adjusted to 500ml by adding 142.5ml water and 57.5ml glacial acetic acid.
Prehybridization buffer for *in situ* mRNA hybridisation (2x) [1]	1.2M NaCl, 20mM Tris-HCl pH 7.5, 2 x Denhardt's solution, 2mM EDTA, 1mg/ml sonicated single-stranded salmon testes DNA, 0.2mg/ml yeast tRNA.
RNase buffer for *in situ* mRNA hybridization	0.5M NaCl, 10mM Tris-HCl pH 7.5 and 1mM EDTA.
SSC (20x) [2]	Mix 175.32g 3M NaCl and 88.23g 0.3M trisodium citrate. Adjust pH to 7 with 10M NaOH and volume to 1L with water.
Storage buffer for nuclei	25% glycerol, 5.0mM Mg acetate, 0.1mM EDTA and 50.0mM Tris-HCl pH 8.0. Just before use 1.0mM DTT and 0.1mM PMSF were added.
5M sodium chloride[2]	292.2g NaCl dissolved to a final volume of 1L.

Solution	Composition
Southern blot hybridization buffer	10% dextran sulphate, 6 x SSC, 1% SDS and 0.1mg/ml salmon testes DNA (denatured for 2min at 100°C before adding to the buffer).
TBE (10x)	108g Trizma base, 55g boric acid, 20ml 0.5M EDTA were dissolved in water to a final volume of 1L.
TE (1x)	10mM Tris-HCl pH 8.0, 1mM EDTA
0.1M triethanolamine[1]	13.3ml triethanolamine was added to 800ml DEPC water, pH was adjusted to 8.0 with HCl then volume was adjusted to 1L with DEPC water.
1M Tris-HCl pH 8.0 or pH 7.5	121.1g Trizma base was dissolved in 800ml water, pH was adjusted with concentrated HCl as required and the volume adjusted to 1L.
Tris-saturated phenol	Phenol was thawed from -20°C with a small amount of H_2O added. Hydroxyquinoline was added to a concentration of 0.1% (w/v) to prevent oxidation and the phenol was equilibrated twice each with 0.5M, 0.2M and 0.1M Tris pH 8. The Tris-saturated phenol was stored at 4°C under a layer of 0.1M Tris pH8.

2.1.5.2 Molecular biology buffers

All buffers were provided by Promega Ltd.

Buffer	Composition
PCR buffer (1x)	20mM Tris-HCl, 50mM KCl
Reverse Transcription Buffer (1x)	10mM Tris-HCl pH 8.8, 50mM KCl, 0.1% Triton X-100.
T4 DNA ligase buffer (1x)	30mM Tris-HCl pH 7.8, 10mM $MgCl_2$, 10mM DTT, 1mM ATP.
Transcription optimised buffer (1x)	40mM Tris-HCl pH 7.9, 6mM $MgCl_2$, 2mM spermidine, 10mM NaCl.

2.1.5.3 Restriction enzyme buffers

All buffers were supplied by Promega Ltd, apart from universal restriction buffer which was prepared in our laboratory.

Buffer	Composition
Universal restriction buffer (1x)	500mM NaCl, 500mM Tris-HCl pH 8.0, 110mM $MgCl_2$, 60mM β-mercaptoethanol, 1mg/ml bovine serum albumin
AvaI/*PvuII* buffer (1x)	6mM Tris-HCl, 6mM $MgCl_2$, 50mM NaCl, 1mM DTT (pH 7.5 at 37°C).
EcoRI/*PstI* buffer (1x)	90mM Tris-HCl, 10mM $MgCl_2$, 50mM NaCl (pH 7.5 at 37°C).
HindIII buffer (1x)	6mM Tris-HCl, 6mM $MgCl_2$, 100mM NaCl, 1mM DTT (pH 7.5 at 37°C).

Buffer	Composition
KpnI/*SmaI* buffer (1x)	10mM Tris-HCl, 7mM $MgCl_2$, 50mM KCl, 1mM DTT (pH 7.5 at 37°C).
NcoI/*SalI* buffer (1x)	6mM Tris-HCl, 6mM $MgCl_2$, 150mM NaCl, 1mM DTT (pH 7.9 at 37°C).
SphI buffer (1x)	10mM Tris-HCl, 10mM $MgCl_2$, 150mM KCl (pH 7.4 at 37°C).

2.1.6 Animals and cells

2.1.6.1 Animals

Wistar rats were supplied by Charles River UK Ltd, Margate, Kent, UK.

2.1.6.2 Bacterial cells

E. coli strains used were HB101 and JM109. Competent JM109 cells were supplied by Promega Ltd.

2.1.7 Bacterial growth media

Medium	Recipe
Luria-Bertoni (LB) broth	1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl.
LB-agar	LB broth with 1.5% (w/v) agar. For pouring culture plates, LB-agar was melted in a microwave, allowed to cool then poured into 10cm petri dishes. If required, ampicillin was added to a concentration of 100μg/ml before pouring.

2.1.8 DNA constructs

2.1.8.1 Primers for use in semi-quantitative RT-PCR.

Primer annealing temperatures are those determined under conditions of 0.1M Na^+.

Primer ID	Sequence	Description	Product length (rat) bp	Product length (mouse) bp
Exon 1	5'-CTTGCTGGAAGTGTCTGGGATG-3'	Exon 1_1, 62°C anneal.	≈200	≈200
T4449	5'-CAAAGCAACACCGTAACACC-3'	Rat exon 1_4, 51.7°C anneal.	463	474
F5984	5'-AAAGGGGCGGGCAGCTTAG-3'	Mouse exon 1_4, 55.3°C anneal.	372	336

Primer ID	Sequence	Description	Product length (rat) bp	Product length (mouse) bp
Exon 1_4	5'-TTGCACCCCAAAGCAACACC-3'	Modified exon 1_4 primer for both species, 62°C anneal.	369	333
Exon 1_5	5'-AAGAGGGTTTTGCATTCG-3'	Exon 1_5, 55°C anneal	364	328
A2955	5'-ACCTGGCGGCACGCGA-3'	Exon 1_6, 53.5°C anneal.	438	405
T5366	5'-AAAGAAACTCGGTTTCCCTC-3'	Exon 1_7, 49.6°C anneal.	379	343
B6251	5'-GTTGACTTCCTTCTCCGTGA-3'	Exon 1_{10}, 51.7°C anneal.	457	430
T4451	5'-CGGCCTTATCTGCTAGAAGT-3'	Exon 1_{11}, 51.7°C anneal.	463	474
Exon 2	5'-CATGGACAGTGAAACGGC-3'	Exon 2, 50.2°C anneal.	n/a	n/a

2.1.8.2 Primers for generation of templates for riboprobes used for *in situ* mRNA hybridisation.

Primer annealing temperatures are those determined under conditions of 0.1M Na^+.

Primer ID	Sequence	Description
Exon 1	5'-TCTGAAACATCTTCCTGGCT-3'	Exon 1_1 3', 58°C anneal.
P17	5'-CCAAAGAGGAGTCACTGTA-3'	Exon 1_1 5', 58°C anneal.
V6895	5'-ATAATACGACTCACTATAGGGCTTTGGAGTCCA TTGGCA-3'	Exon 1_{10} 3', 65.5°C anneal.
W6489	5'-TTAATACGACTCACTATAGGGCCAGCGCGCTCA CACT-3'	Exon 1_5 3', 67.7°C anneal.
W6490	5'-TATTAACCCTCACTAAAGGGTAAGAGGAGGGC GGACT-3'	Exon 1_5 5', 66.6°C anneal.
V6896	5'-TTAATACGACTCACTATAGGGCAGCGTGTGCC GACCT-3'	Exon 1_7 3', 67.7°C anneal.
V6897	5'-CATTAACCCTCACTAAAGGGCACCGTTTCCGTG CAT-3'	Exon 1_7 5', 66.7°C anneal.
Y3011	5'-CTACTACTACTAGGCCACGCGTCGACTAGTAC-3'	Exon 1_{10} 5', 65.6°C anneal.
Y3012	5'-TATTAACCCTCACTAAAGGGAGCGGCGTCTGGA CC-3'	Exon 1_{11} 5', 67.9°C anneal.
Y3021	5'-TTAATACGACTCACTATAGGGCTAGCGCCCAA GTTGTC-3'	Exon 1_{11} 3', 66.6°C anneal.
Z3342	5'-ATAATACGACTCACTATAGGGAATCTGCCTGA GAAGC-3'	Exon 2 3', 64.4°C anneal.
Z3343	5'-AATTAACCCTCACTAAAGGGCCAATGGACTCC AAAGA-3'	Exon 2 5', 64.4°C anneal.

2.1.8.3 Primers for cycle sequencing.

Primer annealing temperatures are those determined under conditions of 0.1M Na^+.

Primer	Sequence	Description
D3720	5'-GATTTAGGTGACACTATAG-3'	SP6 promoter sequencing primer, 44.5°C anneal.
T7	5'-GTAATACGACTCACTATAG-3'	T7 promoter sequencing primer, 52°C anneal.

2.1.8.4 Plasmid constructs

pVL constructs were a kind gift from Mrs Val Lyons in our laboratory. pJIM constructs were a kind gift from James McCormick in our laboratory. pNNCAT was a kind gift from M.D.Jacobson. pGR9 was a kind gift from Mrs June Noble in our laboratory. pTRI-Beta-actin-125-Rat was supplied by AMS Biotechnology.

Plasmid	Vector	Insert
pVL131	pGEM-T	367bp rat 5'-RACE clone containing exon 1_{10}
pVL132	pGEM-T Easy	295bp rat 5'-RACE clone containing exon 1_{11}
pVL133	pGEM-T	228bp rat 5'-RACE clone containing exon 1_4.
pVL135	pGEM-T	290bp rat 5'-RACE clone containing exon 1_5.
pVL154	pGEM3	5-6kb EcoRI fragment from λ208 (rat clone containing exon 2 and approximately 15kb of the rat GR gene flanking the 5' end of exon 2 (M.D.Jacobson, unpublished data), containing exon 1_{11}.
pVL166	pGEM-T Easy	276bp rat 5'-RACE clone containing exon 1_1.
pNNCAT	pUC00c0	2.2kb NcoI fragment from rat GR promoter upstream of exon 1_{10}.
pGR9	pGEM3	674bp PstI-EcoRI fragment of GR cDNA corresponding to exons 5-9.

Plasmid	Vector	Insert
pJIM2	pGEM-T Easy	414bp rat 5'-RACE clone containing exon 1_{10}.
pJIM5	pGEM-T	269bp rat liver RT-PCR product containing exon 1_6.
pTRI-Beta-actin-125-Rat	pTRI	126 bp cDNA fragment of the rat β-actin gene derived from exon 5
pAL1	pGEM-T Easy	430bp Exon 1_1 RT-PCR product from mouse thymus RNA.
pAL3	pGEM-T Easy	250bp Exon 1_5 PCR product from rat genomic clone.
pAL4	pGEM-T Easy	300bp Exon 1_7 PCR product from rat genomic clone.
pAL5	pGEM-T Easy	250bp Exon 1_{10} PCR product from rat 5'-RACE clone.
pAL6	pGEM-T Easy	250bp Exon 1_{11} PCR product from rat 5'-RACE clone.
pAL7	pGEM-T Easy	300bp Exon 2 PCR product from rat 5'-RACE clone.
pAL9	pUC18	250bp *SphI/SalI* recovery of insert from pAL3 (Exon 1_5).
pAL10	pUC18	250bp *SphI/SalI* recovery of insert from pAL6 (Exon 1_{11}).
pAL11	pUC18	300bp *SphI/SalI* recovery of insert from pAL7 (Exon 2).
pAL13	pGEM3	430bp *EcoRI* recovery of insert from pAL1 (Exon 1_1).
pAL14	pGEM3	300bp *EcoRI* recovery of insert from pAL4 (Exon 1_7)
pAL15	pGEM3	250bp *EcoRI* recovery of insert from pAL5 (Exon 1_{10})

2.2 Methods

2.2.1 Animals

2.2.1.1 General husbandry

Animals were maintained under controlled lighting (lights on from 0700 to 1900 hours) and temperature (22°C) with water and food available *ad libitum*.

2.2.1.2 Adrenalectomy

This procedure was performed by Mrs. June Noble (for the short-term adrenalectomy experiment) or Mr. Mark Cleasby (for the long-term adrenalectomy experiment). Anaesthesia was induced and maintained using halothane (Fluothane, Merial) in a Perspex anaesthetic chamber (constructed in-house). Intraoperative analgesia was provided with buprenorphine 0.05mg/kg s/c (Vetergesic, Reckitt & Colman). A 1-2cm transverse incision was made through the skin, subcutis and abdominal musculature approximately 1cm caudal to the last rib, then the adrenal gland was located in the perirenal fat and removed. The abdominal muscle and subcutis were closed with simple continuous sutures of 2/0 braided silk. The skin was closed with stainless steel Michel clips. Sham-operated animals underwent the same procedure, except the adrenal glands were left intact.

2.2.1.3 Short-term (ST) glucocorticoid replacement

4 groups of 6 animals were used to study the effect of short-term (72h) adrenalectomy and subsequent glucocorticoid replacement on GR expression in the hippocampus.

The groups were:

(a) Sham-operated controls that received injections of corn oil vehicle only (Sham).

(b) Adrenalectomised animals that received injections of corn oil vehicle only (Adx).

(c) Adrenalectomised animals that received "physiological" corticosterone replacement (Adx/phys), administered as a daily injection of 0.2mg/kg corticosterone acetate in vehicle at 4pm.

(d) Adrenalectomised animals that received "supraphysiological" corticosterone replacement (Adx/supra), administered as a daily injection of 2mg/kg corticosterone acetate in vehicle at 4pm.

A time-course of 3 days was chosen because previous work from this laboratory had shown significant changes in hippocampal glucocorticoid receptor mRNA levels 2 days after adrenalectomy (Holmes et al., 1995b). Injections of vehicle or corticosterone acetate were given on days 0, 1 and 2. Injections were performed by Mr. Keith Chalmers.

Daily injections of corticosterone acetate were chosen rather than implantation of subcutaneous pellets principally to mimic the natural diurnal afternoon/evening peak of corticosterone release in the rat. Second, corticosterone pellets display rapidly changing release kinetics in the initial period after implantation, with a high initial rate of release falling off sharply to a steady rate of release. The animals would thus be experiencing a variable dose of corticosterone for the majority of the experimental period: this variation was considered a potential source of experimental error.

2.2.1.4 Long-term (LT) glucocorticoid replacement

3 groups of animals were used to study the effect of long-term (3 weeks) adrenalectomy and subsequent glucocorticoid manipulations on GR expression in the hippocampus. The experimental animals were part of a study being performed by Mr Mark Cleasby. For details of experimental procedures see section 2.2.1.

The groups were:

(a) Sham-operated controls (Sham).

(b) Adrenalectomised animals (Adx).

(c) Adrenalectomised animals implanted with pellets containing corticosterone (Adx/cort).

In this case subcutaneous pellets were used to provide corticosterone replacement because the longer experimental period made daily injections impractical. Subcutaneous pellets contained corticosterone 21-acetate in carrier. The corticosterone content of the pellets was calculated to provide a dose of 1mg/kg/day. Sham-operated and vehicle groups received pellets of carrier alone. Pellet implantation was performed by Mr. Mark Cleasby at the time of adrenalectomy.

Trunk blood was obtained for plasma corticosterone assays (section 2.2.13). Animals in both studies were weighed before surgery and again at the time of death.

2.2.1.5 Harvesting of tissues

In both experiments animals were killed by decapitation 1-2h after the beginning of the light period. Trunk blood samples were collected as described in section 2.2.13. The liver, brain and thymus were harvested from both sets of experimental animals. When tissues were harvested care was taken to ensure that an animal was killed from each experimental group in turn to minimise any effect of time of death on GR levels. Tissues were removed, quick-frozen on dry ice and stored at -80°C until required.

2.2.2 Agarose Gel Electrophoresis

2.2.2.1 Analytical DNA gels

These were prepared by mixing solid agarose with 0.5x TBE to give a final agarose concentration of 0.8-2.5% (w/v) depending on the molecular weight of the DNA of interest and boiling in a microwave oven until the agarose was completely dissolved. 50μg/ml of ethidium bromide was added, the solution was thoroughly mixed then the gel was cast using an appropriately-sized gel mould and comb. After setting the gel was placed in a Biorad Sub-Cell GT electrophoresis tank and 0.5x TBE was added until the gel was just submerged. 1kb DNA ladder markers and experimental samples (with 1μl DNA loading buffer added) were then loaded into the wells and

electrophoresed at 100-200V to resolve the DNA fragments. These were visualized on a UV transilluminator at 254nm and imaged using an Appligene Imager. Images were recorded as TIFF files on 1.44Mb floppy disc or on Seikosha video printer paper.

2.2.2.2 Preparative DNA gels

1-1.5% (w/v) agarose gels were used for gel purification of DNA fragments. Low melting point agarose was used and the gel was cast in a cold room at 4°C. Electrophoresis at 150V was performed to separate out the fragment of interest, which was visualized using a UV transilluminator at 365nm to avoid UV light-induced damage to the DNA. The band of interest was excised with a sterile scalpel and the DNA purified (section 2.2.5.2).

2.2.2.3 Analytical RNA gels

Integrity of total RNA was assessed by electrophoresis through a denaturing agarose gel. Gel trays, combs and tank were cleaned with detergent then soaked in 0.1M NaOH for 30min and rinsed several times with ultrapure H_2O before use. 50ml 1.3% (w/v) agarose gels were prepared: 0.6g agarose was dissolved in 36ml DEPC H_2O by boiling in a microwave. After allowing the mix to cool slightly, 9ml 40% (w/v) formaldehyde solution and 2.5ml 10x MEA were added, the solution was mixed thoroughly and the gel was cast. Before use the set gel was aged in a Biorad Sub-Cell GT gel tank in 1x MEA buffer for 15-30min.

2µl of RNA sample was mixed with 2.5µl 40% (w/v) formaldehyde solution, 2.5µl 10x MEA and 10µl deionised formamide. After incubation at 65°C for 15min, 2µl of a 1:5 mix of 10mg/ml ethidium bromide and gel loading buffer was added to the denatured samples. These were loaded on the gel and resolved for 20-30min at 100V. RNA was visualized as described for analytical DNA gels.

2.2.3 Denaturing polyacrylamide gel electrophoresis

Different percentage polyacrylamide gels were used depending on their application. For electrophoresis of cycle sequencing reactions a 6% acrylamide gel was used, for

determination of the quality of cRNA probes a 5% gel was used and in RNase protection analysis a 4% gel was used. Before use, all glass plates were scrubbed with detergent, rinsed in deionised water then rinsed with absolute ethanol and allowed to dry before assembly.

For 6% gels, two 45cm x 35cm glass plates were cleaned, assembled with 0.3mm spacers between them and clamped with bulldog clips. The edges of the plates were sealed with 2-3% (w/v) agarose. 42g urea was dissolved in 15ml 40% acrylamide:bis-acrylamide (19:1), 4ml glycerol tolerant buffer and 600µl 10% (w/v) ammonium persulphate made up to a total volume of 100ml with DEPC water. After filtration through Whatman No. 1 filter paper, 40µl TEMED was added to initiate polymerization and the gel was poured immediately. The flat side of a comb was inserted to allow use of a shark-tooth comb once the gel had set. The gel was left to polymerize overnight, wrapped in Clingfilm to prevent desiccation. Electrophoresis was performed in a Kodak Biomax STS45i vertical tank in 0.8x glycerol tolerant buffer. The gel was prerun at 1800V for 30min, then the wells were flushed with buffer to remove accumulated urea and the samples, denatured in formamide loading buffer at 95°C for 5min, were loaded. The gel was run at 1800V until the bromophenol blue had reached the bottom of the gel. At this point, if reading of sequence far from the sequencing primer position was required, a second loading of sample was performed and the gel run until the bromophenol blue of this loading reached the bottom of the gel. The gel was then transferred to Whatman 3MM paper and dried under vacuum at 80°C using a Biorad 583 gel drier. The dried gel was exposed to Kodak Biomax MR film overnight, then re-exposed for longer if necessary.

For 5% gels a 10-tooth comb and two 8cm x 10cm glass plates were cleaned. The plates were assembled with 1mm spacers. 3.6g urea was dissolved in 1.25ml 40% acrylamide:bis-acrylamide (19:1) and 1ml 10x TBE made up to a total volume of 10ml with DEPC water. 100µl 10% (w/v) ammonium persulphate and 10µl TEMED were added, the gel was immediately poured and the comb was inserted. The gel was allowed to polymerize for at least 30min before use. Electrophoresis was performed

in 1x TBE buffer using a vertical electrophoresis tank. Samples were denatured in formamide loading buffer for 5min at 95°C before loading. The gel was run at 15mA for 20-25min then transferred to Whatman 3MM paper, wrapped in Clingfilm and exposed against either a phosphor screen for 15min or against Kodak Biomax MR film. The phosphor screen was read using a Fujifilm FLA-2000 phosphoimager.

For 4% gels 2 15 x 17cm glass plates were cleaned then assembled with 0.8mm spacers and sealed with 2-3% (w/v) agarose. 21g urea was dissolved in 5ml 40% acrylamide:bis-acrylamide (19:1), 5ml 10x TBE and 300µl 10% (w/v) ammonium persulphate made up to a total volume of 50ml with DEPC H_2O. 100µl TEMED was added to begin polymerization, the gel was immediately poured and a 20-tooth comb was inserted. At least 2h were allowed for polymerization before the gel was used. Electrophoresis was performed in a Model 16 vertical gel electrophoresis system (Life Technologies) in 1x TBE buffer. Wells were flushed before loading of samples (for treatment of samples see section 2.2.11.2). The gel was run at 30mA until the bromophenol blue just ran off the bottom of the gel then was transferred to Whatman 3MM paper and dried under vacuum at 80°C using a Biorad 583 gel drier. The dried gel was exposed to Kodak Biomax MR autoradiography film overnight then developed. The gel was then exposed to a phosphor screen for 1-7d depending on the autoradiography result. If necessary the gel was then reexposed to autoradiography film.

2.2.4 Ribonucleic acid (RNA) extraction

Tissues were obtained from animals killed by decapitation or cervical dislocation (in rats and mice respectively) and immediately snap-frozen on dry ice, then stored at -80°C until required. 50-100mg of tissue was taken, 1ml of TRIzol reagent was added and the tissue was homogenised with an IKA Labortechnik Ultra-Turrax T8 electric homogeniser. Care was taken that the sample did not exceed 10% of the volume of TRIzol reagent used, as use of too much tissue could lead to protein or genomic DNA contamination of the extracted RNA. The homogenised sample was incubated for 5min at room temperature to allow complete dissociation of nucleoprotein complexes, then 0.2ml chloroform was added to each tube. The tube was shaken

vigorously by hand for 15s then incubated at room temperature for 2-3min. The sample was then centrifuged in a microcentrifuge at 12,000g for 15 minutes at 4°C to separate the aqueous and organic phases. The upper aqueous phase was removed to a fresh tube and the RNA precipitated by adding 0.5ml isopropanol and incubating for 10min at room temperature. The RNA was then pelleted by centrifuging the sample at 12000g for 10min at 4°C. The supernatant was carefully removed and the pellet washed by adding 1ml ice-cold 75% ethanol and vortexing. The sample was then centrifuged at 7500g for 5min at 4°C and the supernatant was removed with a drawn-out glass Pasteur pipette. The RNA pellet was air-dried for 5-10min at room temperature until the edges were just translucent, then dissolved in 10-150µl of either DEPC-treated water or 100% deionised formamide (if for use in RNase protection analyses) by heating to 55-60°C for 10min and repeatedly passing through a pipette tip. Samples were then stored at -80°C until required.

2.2.5 Manipulation of DNA

2.2.5.1 Restriction digests

Plasmid DNA (approximately 0.5µg for screening of "minipreps", 5µg for subcloning or linearisation of DNA templates) was digested at 37°C for 60-90min in a mixture containing 1 x restriction buffer (either commercially-bought buffer supplied with the enzyme or our own lab universal restriction buffer) and 5-15U of restriction endonuclease made up to a final volume of 15-30µl with ultrapure H_2O. Digestion was verified by agarose gel electrophoresis of the whole digest (for minipreps) or a 1µl aliquot.

Genomic DNA was digested at 37°C for 12-18h in a similar mixture with a final volume of 50-100µl. A second aliquot of 5-15U restriction endonuclease was added after approximately 12h of incubation.

If the DNA fragment was for ligation and both the insert and vector had been cut with the same enzyme (generating an overhanging end), an equal volume of TE and 1U calf intestinal phosphatase was added followed by incubation for a further 60min at 37°C then 15min at 65°C to inactivate the phosphatase. If a blunt 5' end was

required for ligation this was created from a 5' overhang by adding 5U Klenow fragment and 1/10 vol 2mM dNTPs then incubating at 37°C for 10min followed by 65°C for 10min.

2.2.5.2 Purification of digested plasmid DNA

For subcloning, DNA fragments of greater than 200bp in size were purified using the Hybaid Recovery DNA Purification Kit II. Fragments were resolved by electrophoresis at 120V on a 1.5% low melting point agarose gel. The gel was examined on a UV transilluminator at 365nm (to avoid DNA damage) to identify the bands of interest, which were then excised. Linearized plasmid DNA was purified directly from the restriction digest reaction. Either the entire restriction digest or the excised gel slice was placed in a spin filter tube to which 400µl of the supplied binding buffer had been added. The mixture was incubated at 55°C for 5min, agitated then centrifuged in a microcentrifuge at 14000rpm for 30s. Two washes were performed with 500µl of supplied Wash Solution being added each time followed by centrifugation as before. The pellet was dried by centrifuging for 1min, then the purified DNA was eluted into a fresh catch tube by adding 15-25µl of Elution Solution, vortexing briefly then centrifuging as before.

DNA fragments of less than 200bp were recovered using the High Pure PCR Product Purification Kit. Fragments were resolved by electrophoresis and gel slices were excised as described above. Gel slices were placed in a preweighed Eppendorf tube and weighed. 300µl of supplied binding buffer per 100mg agarose was added, the sample was vortexed and heated to 56°C for 10min, vortexing every 2-3min. After the agarose had completely dissolved, 150µl isopropanol/100mg agarose was added and the mixture was vortexed thoroughly. The entire mixture was then placed in the High Pure filter tube and centrifuged for 30-60s in a microcentrifuge at maximum speed. The flowthrough solution was discarded and 500µl supplied wash buffer was added to the filter. The tube was centrifuged as above, the flowthrough was discarded and the wash step was repeated with 200µl wash buffer. The filter was attached to a fresh collecting tube, 50-100µl supplied elution buffer was added to the filter and the

tube was centrifuged as above. The eluted DNA was retained and stored at -20°C until required.

In both cases, DNA recovery was checked by electrophoresis of a 1µl aliquot on a 1-1.5% agarose gel.

2.2.6 DNA cloning

2.2.6.1 Ligation of DNA

DNA fragments were purified and recovered as described in Section 2.2.5.2, then ligated in a mixture containing 1 x ligation buffer, 1-5U T4 DNA ligase, approximately 50ng insert DNA and approximately 100ng vector DNA. The mix was incubated at 4°C overnight then used for transformation of bacterial cells (section 2.2.6.3).

2.2.6.2 Preparation of competent bacterial cells

For cloning of PCR products, supercompetent *E.coli* JM109 were purchased. For other cloning procedures the *recA*-HB101 strain of *E.coli* was used. Transformation-competent cells of this strain were produced as follows. 1ml of LB was inoculated with a single colony of cells and incubated in a shaking incubator at 37°C overnight, then used to inoculate 50ml of LB. This was incubated as before for 1.5-2h until the cells were in the late log phase of multiplication. The culture was centrifuged in a Beckman J2-MC centrifuge with a JA-14 rotor at 4700g for 5min. Pelleted cells were resuspended in 20ml ice-cold 0.1M $CaCl_2$, incubated for 10min-2h on ice, centrifuged as before then resuspended in 2ml ice-cold 0.1M $CaCl_2$. Competent cells thus prepared were stored on ice at 4°C for up to 4d with daily changes of ice.

2.2.6.3 Transformation of bacterial cells

Supercompetent JM109 cells were transformed as follows. Cells were allowed to thaw from -80°C on ice, mixed gently then 100µl of suspension was transferred to a prechilled 1.5ml Eppendorf tube. 200-500ng of DNA was added, mixed gently then

incubated on ice for 10min. Cells were then subjected to 50s heat shock at 42°C followed by 2min recovery on ice. 400µl of LB broth at room temperature was added and the cells were placed in a shaking incubator at 37°C for 30min. 200µl of suspended cells was plated on a LB agar plate containing 40µl 50mg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-GAL), 40µl 0.1M isopropyl-ß-thiogalactopyranoside (IPTG) and 100µg/ml ampicillin. Plates were allowed to dry for 10min before incubation at 37°C overnight.

2.2.6.4 Screening of transformants: "minipreps"

Single transformant colonies were picked and inoculated into 2ml LB containing 100µg/ml ampicillin then incubated at 37°C in a shaking incubator overnight. Cultures were decanted into 1.5ml Eppendorf tubes then centrifuged at 14000rpm for 1min in a microcentrifuge. Pelleted cells were resuspended in 100µl GTE, 200µl fresh alkaline SDS was added, then the tubes were vortexed and placed on ice for 5min. 150µl 5M potassium acetate pH 4.8 was added, then the tubes were vortexed and placed on ice for 5min. Tubes were centrifuged for 5min as before and the supernatant was removed to fresh Eppendorf tubes containing 225µl chloroform:isoamyl alcohol (24:1) and 225µl Tris-saturated phenol. Tubes were vortexed and centrifuged for 2min as above. The supernatant was transferred to fresh Eppendorfs containing 2 vol ethanol, vortexed and incubated at room temperature for 5min. Tubes were centrifuged for 5min as above, the supernatant was completely removed and the DNA pellet was allowed to air dry for 10min. The DNA was resuspended in 50µl TE containing 50ng RNase A and stored at -20°C. Appropriate restriction enzyme digests and agarose gel electrophoresis were performed to identify colonies containing plasmids with the desired insert.

2.2.6.5 Preparation of large quantities of plasmid DNA

A single transformed colony was used to inoculate 2ml of LB broth containing 100µg/ml ampicillin then incubated overnight at 37°C in a shaking incubator. This culture was used to inoculate 500ml of LB broth containing 100µg/ml ampicillin which was incubated overnight at 37°C in a shaking incubator. Cells were pelleted by centrifugation for 5min at 3500g and 4°C in a Beckman J2-MC centrifuge with a JA-

14 rotor, then resuspended in 12ml GTE. 24ml of fresh alkaline SDS was added and the mix incubated on ice for at least 10min. 16ml cold 5M potassium acetate was added. The sample was mixed thoroughly, incubated on ice for 10min then centrifuged for 10min as above. The supernatant was filtered through gauze into fresh centrifuge pots containing 32ml isopropanol and left at room temperature for 30min. DNA was pelleted by centrifugation for 3min at 9600g and 4°C. The pellet was briefly air-dried then resuspended in 2.2ml TE. 3g of CsCl and 100µl of ethidium bromide was added then the solution was transferred into 3ml Beckman Quickseal tubes and centrifuged for either 16-20h at 70000rpm or 4h at 100000rpm in a Beckman Optima TLX ultracentrifuge using a TLA100.3 rotor. The band of ethidium-stained plasmid DNA was removed and transferred to a fresh tube using a sterile needle and syringe. TE with 1g CsCl added per ml TE was used to fill the tube and the centrifugation was repeated. The ethidium-stained DNA was removed as above and repeated isopropanol extractions were performed to remove the ethidium bromide. The DNA solution was then dialysed overnight against 3 changes of 2L TE solution at 4°C. Concentration and quality (measured by A_{280}/A_{260} absorbance ratio) of the recovered DNA was determined using a Pharmacia Biotech GeneQuant spectrophotometer. Samples were then stored at -20°C.

2.2.7 Polymerase Chain Reaction techniques

2.2.7.1 Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Single-stranded cDNA was synthesized using the Reverse Transcription System kit (Promega). Reactions contained 5mM $MgCl_2$, 1 x reverse transcription buffer, 1mM each dNTP, 40U RNasin ribonuclease inhibitor, 30U AMV reverse transcriptase, 1µg oligo$(dT)_{15}$ primer, 2µg total RNA and nuclease-free water to a final volume of 40µl. Control reactions were identical in all respects except that no reverse transcriptase was added. Reactions were incubated at 42°C for 30min, 95°C for 5min then 4°C for 5min. Products were stored at -20°C until required. 2µl of either neat or diluted (1/5, 1/25, 1/125 in DEPC water) product was used in a PCR reaction containing 1 x PCR buffer, 1.5mM $MgCl_2$, 200µM each dNTP, 40pM each primer

(section 2.1.8) and nuclease-free water to a final volume of 50µl. Reactions were also set up for each primer pair containing either 2µl control reaction without reverse transcriptase or 2µl nuclease-free water. 1 TAQbead (Promega) was added to each tube followed by 2 drops of mineral oil to prevent evaporation. PCR amplification (95°C 5min followed by 35 cycles of 95°C 50s, 50°C 45s, 72°C 90s then 72°C for 5min) was performed in an Eppendorf Mastercycler Gradient thermal cycler. A 10µl aliquot of each product was run on a 1.5% analytical agarose gel and amplified DNA fragments were visualized as described in section 2.2.2.1.

2.2.7.2 Cycle sequencing

This was performed using the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham). Reactions were prepared containing 2µl dGTP nucleotide master mix, 0.5µl [α^{33}P]-dNTP (A, C, G or T with only 1 being added per sequencing reaction) and 4.5µl reaction master mix (2µl reaction buffer, 1µl DNA solution, 0.05pM primer (section 2.1.8.3), 13µl ultrapure water and 8U Thermo Sequenase polymerase). Tubes were placed on a preheated thermal cycling block and 40 cycles of PCR amplification (95°C 30s, 45°C 30s, 72°C 1min) were performed. 4µl of stop solution was added and the reactions were resolved on a 6% glycerol tolerant sequencing polyacrylamide gel.

2.2.7.3 General PCR protocol

A reaction mix was prepared containing 1 x PCR buffer, 0.5mM $MgCl_2$, 40pM each primer (section 2.1.8), 200µM each dNTP and approx. 100ng of DNA template. The reactions were overlaid with 2 drops of mineral oil and denatured at 95°C for 5min before adding 2.5U *Taq* DNA polymerase and beginning cycling. Appropriate cycling conditions were used for the primer pair in use (section 2.1.8).

2.2.8 Southern blot analysis

2.2.8.1 Restriction enzyme digestion

50µg of genomic DNA was digested overnight as described in section 2.2.5.1. Enzymes used are described in Chapter 5.

2.2.8.2 Electrophoresis of samples and transfer to nylon membrane

After restriction enzyme digestion DNA samples were electrophoresed overnight at 15-20V on a 0.8% analytical agarose gel as described in section 2.2.2.1. Towards the end of the running time the DNA was visualised as described in section 2.2.2.1 to ensure that the smallest fragments of DNA were not lost from the end of the gel. Gels were treated by shaking in 0.125M HCl for 15min, 1.5M NaCl/0.5M NaOH for 30min and 1.5M Tris pH 7.5/1.5M NaCl for 30min on a Bellco Biotechnology orbital shaker. DNA was transferred to a positively-charged nylon membrane (Hybond N Plus, Amersham) by capillary transfer. The gel was inverted and placed on a wick of Whatman 3MM paper soaked in 20 x SSC. The nylon membrane was applied to the gel, with care being taken to ensure no air bubbles lay between it and the gel. 3 layers of Whatman 3MM paper cut to the same size as the membrane and a 2-3 inch layer of paper towel (Wypall, Kimberley-Clark) folded to the same size as the membrane were layered on top. A glass plate was applied to the top of the towel stack and weighted with approximately 150g. The plate was levelled with a spirit level, then the whole assembly was covered with Clingfilm and left at room temperature overnight. The towels etc. were then removed and the blot was washed in fresh 20 x SSC to remove any agarose sticking to it. The blot was allowed to air dry, then the DNA was crosslinked to the nylon membrane by exposing it to 120mJ/cm^2 of ultraviolet radiation in a "Spectrolinker" XL-1500 ultraviolet crosslinker (Spectronics Co.).

2.2.8.3 Preparation of cDNA probe

Probes were prepared using the Random Primed DNA Labeling Kit (Roche Diagnostics). 1-2μg of DNA template was denatured at 100°C for 10min, then cooled on ice for 5min. A reaction mixture was prepared containing this DNA, 75μM each of dATP, dGTP and dTTP, 1 x reaction mixture containing hexanucleotide primers (supplied with kit), 1.85MBq [α^{32}P]-dCTP, 2U Klenow fragment of DNA polymerase I and H_2O to a final volume of 20μl. The reaction was incubated at 37°C for 60-90min then the probe was purified using a Sephadex G-50 grade ("Nick") column. The column was first equilibrated with 3ml TE, then the reaction mix was

added and allowed to soak into the gel bed. 400µl TE was added and the eluate discarded. A further 2 elutions were performed with 200µl of TE each and the eluates collected. The specific activity of probe in the eluates was assayed by adding a 1µl aliquot to 1ml of Aquasafe 300 Plus scintillant fluid and counting on a Wallac 1450 Microbeta Plus scintillation counter. The eluate with the highest activity was retained for use in hybridisation and stored at -20°C for a maximum of 48h until required.

2.2.8.4 Hybridization

This was performed in glass hybridisation bottles in a Techne Hybridizer HB-1D hybridisation oven. Two methods of hybridisation were used, employing different buffers. In the first method, the blot was prehybridized for 3-4h at 65°C in 20-25ml of Southern blot hybridization buffer. The probe was denatured at 95°C for 10min and immediately added to the hybridisation bottle. The volume of buffer used was calculated so that the final specific activity of probe would be approximately $1x10^6$cpm/ml. The blot was hybridised for 14-16h, then the buffer was removed and the blot was quickly washed in room temperature 2 x SSC followed by 2 washes of 15min in 0.1 x SSC/1% SDS at 65°C. In the second method the blot was prehybridized at 42°C in a commercially-available hybridisation buffer (Ultrahyb, Ambion Inc.). The probe was denatured and added as above, then the blot was hybridised for 14-16h at 42°C. The buffer was removed and the blot was washed for 2 x 15min in 2 x SSC/0.1% SDS at 42°C, then for 2 x 15min in 0.1 x SSC/0.1% SDS at 42°C. In both cases after washing the blot was wrapped in Clingfilm, examined with a Geiger-Muller counter and exposed to a phosphor screen for 2-8h depending on the amount of radioactivity detected. The bands were visualized using a Fujifilm FLA-2000 phosphoimager. The blot was then exposed to autoradiography film for 1-14d depending on the strength of signal observed on the phosphoimager.

2.2.9 Northern blot analysis

2.2.9.1 Electrophoresis of samples and transfer to nylon membrane

RNA samples were electrophoresed at 150V on a 1.3% analytical RNA gel as described in section 2.2.2.3. The gel was run until the bromophenol blue dye front was 1-2cm from the end of the gel. RNA was transferred to a positively-charged nylon membrane (Hybond N Plus, Amersham) as described for Southern blot analysis in section 2.2.8.2.

2.2.9.2 Hybridization

This was performed in glass hybridisation bottles in a Techne Hybridizer HB-1D hybridisation oven. Blots were prehybridised at 55°C in Northern blot hybridisation buffer (section 2.1.5.1) for at least 3h before probes, prepared as described in Section 2.2.8.3, were denatured for 10min at 95°C and immediately added to the bottle. Hybridization was performed for 14-16h at 55°C. After hybridisation the buffer was removed and the blot was washed for 3 x 20min in a solution of 0.1 x SSC/0.1% SDS at room temperature. The radioactivity levels on the blot were assessed with a Geiger-Muller counter and if necessary the blot was then washed for 30min in a solution of 0.3 x SSC/0.1% SDS at 55°C. From this point the blot was treated as described for Southern blot analysis in section 2.2.8.4.

2.2.10 In situ mRNA hybridization

2.2.10.1 Preparation of slides

Glass microscope slides were prepared by submersion in 0.2M hydrochloric acid for 3min, submersion for 3min in DEPC H_2O, dipping in 2% silane in acetone, submersion for 2 x 3min in acetone then submersion for 3min in DEPC H_2O. Slides were briefly air-dried at room temperature then baked at 50°C overnight in a size 2 Gallenhamp Hotbox oven.

2.2.10.2 Sectioning of tissues

Tissues were cut at -20°C into 15μm sections using a Bright Model OTF cryostat. Tissues were removed from storage at -80°C and mounted for sectioning using Cryo-

M-Bed embedding compound, then allowed to warm to -20°C for 15-20min. Glass microscope slides prepared as described above were pre-chilled to -20°C before use. 3 sections of tissue were collected onto each slide and fixed in place by brief melting. Sections were stored at -80°C until required.

2.2.10.3 Preparation of cRNA probes

Probes for *in situ* hybridization were prepared using either PCR products or their subclones as templates. Initially, DNA templates for the synthesis of cRNA probes for exons 1_5, 1_7, 1_{10}, 1_{11} and 2 were generated by PCR amplification from various 5'-RACE and genomic clones using exon-specific primers (Table 2.1: for details of primer positioning on the genomic sequence see section 2.1.8.2 and Appendix A, for details of 5'-RACE and genomic clones see 2.1.8.4). All these PCR product templates used phage T7 polymerase to generate the antisense probe: all but the 1_{10} template (whose 5' primer had no phage polymerase recognition sequence) used phage T3 polymerase to generate the sense probe.

Exon(s)	Plasmid template	5' primer	3' primer	Product length
2	pVL131	Z3343	Z3342	200bp
1_5	pNNCAT	W6490	W6489	228bp
1_7	pNNCAT	V6897	V6896	290bp
1_{10}	pVL131	Y3011	V6895	290bp
1_{11}	pVL154	Y3012	Y3021	290bp

Table 2.1: primers and plasmid constructs used to generate PCR product templates for cRNA probes.

The probe for exons 5-9 was generated from plasmid construct pGR9 (section 2.1.8.4) linearised with *AvaI* to generate the antisense probe and *EcoRI* to generate the sense probe.

Subsequently, plasmid constructs pAL2 to pAL7 (section 2.1.8.4) were created by subcloning the PCR product templates into the pGEM-T Easy vector system. Also, plasmid construct pAL1 (section 2.1.8.4) was generated by subcloning a RT-PCR product (generated from mouse thymus RNA using primers P17 and 1_1) into the same vector. Orientation of inserts was determined by cycle sequencing (section 2.2.7.2). Plasmid templates were linearised with the appropriate enzyme and probes transcribed with phage T7, T3 or SP6 RNA polymerase (Table 2.2).

Further subcloning was undertaken into the pUC18 and pGEM3 vectors to create plasmids pAL9 to pAL15 (section 2.1.8.4). Orientation of inserts was determined and probes were generated as described in the preceding paragraph.

Exon	Plasmid	Restriction enzymes/phage polymerase
1_1	pAL1	*NcoI*/SP6 AS, *SalI*/T7 S
1_5	pAL3	*SalI*/T7 AS, *NcoI*/T3 S
1_7	pAL4	*SalI*/T7 AS, *NcoI*/T3 S
1_{10}	pAL5	*SalI*/T7 AS, *SphI*/SP6 S
1_{11}	pAL6	*SalI*/T7 AS, *NcoI*/T3 S
2	pAL7	*NcoI*/T7 AS, *SalI*/T3 S
1_5	pAL9	*SalI*/T7 AS, *HindIII*/T3 S
1_{11}	pAL10	*SalI*/T7 AS, *HindIII*/T3 S
2	pAL11	*HindIII*/T7 AS, *SalI*/T3 S
1_1	pAL13	*SmaI*/T7 AS, *PvuII*/SP6 S
1_7	pAL14	*PvuII*/SP6 AS, *SmaI*/T3 S
1_{10}	pAL15	*PvuII*/SP6 AS, *HindIII*/T7 S

Table 2.2: restriction enzymes and phage polymerases used with plasmid templates for generation of RNA probes. AS = antisense, S = sense.

A reaction was set up containing 1x transcription optimized buffer, 0.3mM each of ATP, CTP and GTP, 5.92MBq [α^{35}S]-UTP, 10mM DTT, 40U RNasin RNase inhibitor, approximately 1µg linearised DNA template and 15U of the appropriate phage polymerase. The reaction was incubated for 60-90min at 37°C (40°C if phage SP6 polymerase was being used), then 1U of RNase-free DNase was added and the mixture was incubated for a further 15min at 37°C. Probes were purified using Sephadex G-50 grade ("Nick") columns. The column was first equilibrated with 3ml TE, the reaction mix was then added and allowed to soak into the gel bed. 400µl TE was added and the eluate discarded. A further elution was performed with 400µl of

TE and the eluate collected. The activity of probe in this eluate was measured (section 2.2.8.3) and the integrity of the probe was verified by electrophoresing $1x10^5$ cpm of probe on a 5% denaturing polyacrylamide gel (section 2.2.3).

2.2.10.4 Fixation of tissues

All solutions for fixation were prepared using sterile glassware and DEPC H_2O. Sterile glass troughs were used for all incubations. Experimental slides were fixed by 10min in 10% paraformaldehyde in phosphate buffer at 4°C, 2 x 5min washes in 1 x PBS, 10min in a solution of 0.25% acetic anhydride and 0.1M triethanolamine, 5min in 1 x PBS then dehydration by passing through 70%, 80% and 95% ethanol for 2min each. Slides were briefly air dried before use.

2.2.10.5 Hybridization

Hybridization was performed in Perspex hybridization boxes whose bases were lined with a double layer of Whatman 3MM paper soaked in box buffer. Sections were prehybridized in 200µl of 1 x prehybridization solution then incubated for 2-3h at 50°C in a size 2 Gallenhamp Hotbox oven. Sections were then incubated at 50°C for 16h in 200µl of 1x hybridization solution containing $1x10^6$ cpm probe. cRNA probes were denatured for 10min at 75°C before adding to the hybridization mix. Slides were washed for 3 x 5min in 2 x SSC then coated with 200µl of RNase buffer containing 30µg/ml RNase A. Slides were incubated at 37°C for 1h in Perspex hybridization boxes lined with a single layer of Whatman 3MM paper soaked in RNase buffer. Washes were performed in a Hybaid Omnislide wash module. Slides were washed for 1h in 2 x SSC at room temperature, 1h in 0.1 x SSC at 60°C and 1h in 0.1 x SSC cooling to room temperature from 60°C. Slides were dehydrated by passing through 50%, 70% and 90% ethanol in ammonium acetate for 2min each, then air dried.

2.2.10.6 Autoradiography

Slides were exposed to a Fuji phosphor screen for 24h and visualized in a Fujifilm FLA-2000 phosphoimager to ascertain if the hybridisation had been successful.

Slides were then exposed to Kodak Biomax MR autoradiography film for 7-14d depending on the strength of signal observed on the phosphoimager. Slides were then dipped in a 50% solution of Kodak NTB2 photographic emulsion, air dried and kept at 4°C for 2-6 weeks depending on the strength of signal observed on the autoradiography film. Slides were developed by immersion for 4min in a 1:1 dilution of Kodak D19 developer and water, followed by 10s in water, 5min in a 1:4 dilution of Amfix High Speed Fixer and water then 5min in water. Slides were then air-dried before staining.

2.2.10.7 Staining of slides

Sections were stained with either haematoxylin and eosin (for liver and thymus) or pyronin (for brain). Haematoxylin and eosin staining was performed by immersing slides for 80s in Meyer's haematoxylin, 20s in H_2O, 1min in alkaline H_2O, 1min in H_2O, 1min in 70% ethanol, 1min in eosin, 2 x 1min in 95% ethanol, 2 x 1min in 100% ethanol then 2 x 1min in Histoclear solution. Pyronin staining was performed by immersing the slides for 3min in 1%(w/v) pyronin (filtered through Whatman No. 1 filter paper before use), washing in deionised water then immersing for 2min in acetone, 2min in 50% acetone/50% xylene and 2 x 2min in xylene. After staining slides were mounted using DPX mounting fluid and glass cover slips, then allowed to dry overnight. Stained emulsion was removed from the non-section side of the slides by scraping with a scalpel.

2.2.10.8 Data analysis

Autoradiographs were examined on a Northern Light B95 Precision Illuminator (Imaging Research Ltd, St. Catharine's, Ontario, Canada) with a MTI CCD725 video camera. 2D densitometry was performed using MCID-M4 version 3.0 (revision 1.5) software (Imaging Research Ltd).

After dipping in photographic emulsion and staining, sections were examined using a Carl Zeiss GmbH Axioskop microscope with a Zeiss Plan-Neofluar 40x objective lens and a Zeiss 10x eyepiece lens. Images were captured using a JVC 3-CCD C-

mount video camera and silver grains counted using Zeiss KS300 v3.0 image analysis software.

Statistical analysis was performed by 1-way ANOVA and Tukey's HSD Test or Fisher's LSD Test using the Statistica software package (version 5), with $p<0.05$ taken as indicating significance.

When examined microscopically, the emulsion-dipped slides from the experiment investigating expression of exon 1_5 in the hippocampus showed marked variability in the number of silver grains present. Approximately 50% of the slides had virtually no silver grains present while the other 50% had comparable amounts to those seen with the other exon 1 probes. The most likely cause for this was a fault in the developing process which rendered it impossible to perform grain counting analysis for this experiment.

2.2.11 Ribonuclease Protection Analysis

2.2.11.1 Synthesis of cRNA probes

Templates for cRNA synthesis were 5'-RACE clones previously generated in this laboratory (section 2.1.8.4), except for the rat β-actin template (pTRI-Beta-actin-125-Rat, section 2.1.8.4) which was supplied by AMS Biotechnology and the exon 1_6 template (pJIM5) which was a subcloned RT-PCR product generated from total rat liver RNA in this laboratory. The templates were linearised with *NcoI* and transcribed with the phage SP6 RNA polymerase, except for the actin probe where the template was linearised by the manufacturer using *XbaI* and *HindIII*. The predicted sizes of protected fragments containing both exon 2 and the specific exon 1 of interest are shown in Table 2.3 along with the predicted size of the rat β-actin protected fragment.

Probe	Plasmid template	Size of protected fragment (nucleotides)
1_1	pVL166	228
1_5	pVL135	242
1_6	pJIM5	221
1_{10}	pJIM2	306
1_{11}	pVL132	247
β-actin	pTRI-Beta-actin-125-Rat	126

Table 2.3: predicted sizes of cRNA probe fragments protected by GR mRNA containing alternate exons 1 in RNase protection analysis.

For probe synthesis a reaction was prepared containing approximately 1μg linearised DNA template, 1x transcription optimised buffer, 0.26mM ATP/CTP/UTP, 3.5mM DTT, 20U RNasin RNase inhibitor and 0.5μl 10mg/ml bovine serum albumin. 5μl of [α^{32}P]-GTP (3000Ci/mmol) was added followed by 20U of the appropriate polymerase. After incubation for 2h at 37°C, 1U of RNase-free DNase was added and the mixture was incubated for a further 15min at 37°C. cRNAs were purified using Sephadex G-50 grade ("Nick") columns. The column was first washed then equilibrated with 3ml TE, then the reaction mix was added and allowed to soak into the gel bed. 400μl TE was added and the eluate discarded. A further elution was performed with 400μl of TE and the eluate collected. The radioactivity of probe in this eluate was measured (section 2.2.8.3) and the integrity of the probe was verified by electrophoresing $1x10^5$ cpm of probe on a 5% denaturing polyacrylamide gel (section 2.2.3). Several bands (corresponding to truncated products) were often

observed on autoradiography, probably due to the high GC content of the DNA templates causing "stalling" and dissociation of the phage polymerase. Probes were only used in assays if the full-length product was the dominant band on the autoradiograph.

2.2.11.2 Ribonuclease protection analysis

These were performed using the HybSpeed RPA Kit (Ambion). In all assays 2 cRNA probes were used, one for actin mRNA (as an internal control) and one for the exon 1 of interest. 50μg of either total RNA (experimental samples) or yeast tRNA (positive or negative controls) was coprecipitated with $1x10^5$ cpm cRNA probe by adding 1/10 vol 5M ammonium acetate and 2.5 vol ethanol then placing at -20°C for 20min. The coprecipitate was pelleted by centrifugation in a microcentrifuge for 15min at 4°C. The supernatant was carefully removed with a drawn-out Pasteur pipette and the pellet resuspended in 20μl hybridisation buffer (provided in kit) by incubating at 95°C and vortexing each sample for 3x10s. Successful resuspension was confirmed by pipetting each sample and holding it next to a Geiger-Muller counter: if ≥ 70% of the total counts were in the pipette tip then resuspension was regarded as satisfactory. Samples were incubated for 2min at 95°C then quickly transferred to 68°C for 1h. 100μl of a 1/25 dilution of a RNase A/T1 mix was added to all tubes except the positive controls which received 100μl digestion buffer without RNase A/T1. Tubes were vortexed, incubated at 37°C for 15min, vortexed again then incubated at 37°C for a further 15min. 150μl of the supplied RNase inactivation/precipitation mix was added, then tubes were vortexed and placed at -20°C for 20min. RNA was pelleted by centrifugation in a microcentrifuge at 14000rpm for 15min then resuspended in 8μl of the supplied gel loading buffer. Resuspension was verified as detailed above. Samples were then electrophoresed on a 4% denaturing polyacrylamide gel: positive control samples were diluted with additional loading buffer so that the amount of radioactivity loaded on the gel was comparable to that loaded in the experimental samples. As a typical assay required the use of 2 polyacrylamide gels, half of the samples from each experimental group were loaded on each gel.

2.2.11.3 Data analysis

Dried polyacrylamide gels were exposed to autoradiography film overnight then to a phosphoimager screen for 1-7d depending on the strength of signal on the corresponding autoradiograph. Quantitative analysis of bands was carried out using a Fujifilm FLA-2000 phosphoimager and Aida v2.0 software (Raytest GmbH) by enclosing them within a rectangle and measuring the intensity of signal within this area by 2D densitometry. Background for each band was determined by placing a similar rectangle just above it. The background value was subtracted and the values for each band corrected for the differences in size of the protected fragment. Each exon 1 probe contains a section complementary to the 5' end of exon 2, so is predicted to give 2 protected fragments, one representing the exon 1 and exon 2 and one representing exon 2 alone. The abundance of each exon 1 was expressed as a proportion of total GR mRNA transcripts and as an absolute value relative to actin:

$$\%\text{total GR mRNA} = \frac{\text{exon 1 \& 2 band intensity}}{(\text{exon 1 \& 2 band intensity}) + (\text{exon 2 band intensity})} x100\ \%$$

$$\text{Absolute quantity of exon 1 - containing GR mRNAs} = \frac{\text{exon 1 \& 2 band intensity}}{\text{actin band intensity}}$$

The abundance of total GR mRNA transcripts was expressed as an absolute value relative to actin:

$$\text{Absolute quantity of GR mRNA} = \frac{\text{exon 2 band intensity}}{\text{actin band intensity}}$$

Statistical analysis was performed by 1-way ANOVA and Tukey's HSD Test or Fisher's LSD Test using the Statistica software package (version 5), with $p<0.05$ taken as indicating significance.

2.2.12 DNase I hypersensitive site mapping

2.2.12.1 Preparation of nuclei

All buffers, centrifuge rotors and other equipment were chilled to 4°C before use and experimental samples were kept on ice throughout the procedure. Liver tissue was

finely chopped while still frozen, rinsed in 0.1M NaCl, 10mM Tris pH 8.0 and homogenised in 10ml Buffer I/g tissue using a Wheaton 15ml glass homogeniser and loose-fitting pestle. The homogenate was filtered through gauze and then homogenised again with a tight-fitting pestle. Lysis of hepatocytes was verified by microscopic examination of 25µl of homogenate. The homogenate was then diluted by addition of 1.6 volumes of Buffer II. Nuclei were purified by density gradient centrifugation through Buffer II in a Beckman J2-MC centrifuge with a JA-21 rotor at 36000G for 45min at 4°C. Supernatant was removed by suction then nuclei were resuspended in 0.5ml storage buffer per pellet. A 1:200 dilution of the nuclei suspension in storage buffer was counted using an Improved Neubauer Haemocytometer and the suspension diluted to give a final concentration of $1x10^7$-$1x10^8$ nuclei/ml. Prepared nuclei were used immediately.

2.2.12.2 DNase I digestion

DNase I digestions were performed on ice. 15ml Corex tubes were prepared, each containing 0-200U of DNase I in a total volume of 60µl 1 x PBS. 300µl of nuclei suspension was added, mixed and left to incubate for 10min on ice. The reaction was stopped by adding EDTA to 20mM. Nuclei were pelleted by centrifugation at 600 x g in a Heraeus Labofuge 400R centrifuge at 0°C. The supernatant was carefully removed and the nuclei resuspended in 1.9ml 50mM Tris pH 8.0, 10mM EDTA. SDS was added to a final concentration of 5% and proteinase K to a final concentration of 100µg/ml. The samples were incubated at 37°C overnight then the genomic DNA was purified.

2.2.12.3 Purification of genomic DNA

Genomic DNA was purified by phenol/chloroform extraction. An equal volume of Tris-saturated phenol was added to the sample and mixed by inversion for 1 minute. The sample was centrifuged for 5min at 1500g in a Heraeus Labofuge 400R centrifuge and the supernatant transferred to a fresh tube, taking care to avoid the interface. This procedure was repeated once more with an equal volume of Tris-saturated phenol, then with 0.5 vol of Tris-saturated phenol and 0.5 vol of chloroform:isoamyl alcohol (24:1), then with an equal volume of chloroform:isoamyl

alcohol. 1/10 vol of 5M NaCl and 2.5 vol of ethanol were added to the final supernatant which was then placed at -20°C overnight. The DNA was pelleted by centrifuging at 3900g in a Heraeus Labofuge 400R centrifuge for 25min at 0°C. The supernatant was completely removed and the pellet briefly air-dried. The pellet was resuspended by adding 0.5-2ml TE (depending on DNA yield) and standing at 4°C for 3-4 d, gently mixing daily. Integrity of the recovered DNA was checked by electrophoresis on a 0.8% analytical agarose gel. Only samples where agarose gel electrophoresis showed no evidence of an excessive amount of low-Mr DNA fragments were used in subsequent experiments.

2.2.13 Plasma corticosterone assay

Trunk blood samples were collected immediately after decapitation into 15ml universal tubes containing 1ml 5% (w/v) EDTA solution. Samples were placed on ice, centrifuged for 10min at 1200g and 4°C in a Heraeus Labofuge 400R centrifuge to remove blood cells. Plasma was removed into fresh 1.5ml Eppendorf tubes and stored at -80°C until required. For the assay, 25µl aliquots of plasma were diluted with 225µl borate buffer and denatured at 75°C for 30min. A mixture of 2µl [1,2,6,7]^{3}H-corticosterone (2.59TBq/mmol), and 9ml borate buffer was prepared: the specific activity of 50µl of this was measured (section 2.2.8.3) and the concentration of ^{3}H-corticosterone adjusted to give a final activity of approximately 2x10^{3}cpm/µl. Rabbit anti-corticosterone antiserum (raised by and a gift from Dr C.Kenyon in our laboratory) was added to a final dilution of 1 in 10000. Standard solutions of corticosterone 21-acetate were prepared to concentrations of 320nM, 160nM, 80nM, 40nM, 20nM, 10nM, 5nM, 2.5nM, 1.25nM, 0.625nM and 0nM. 50µl of ^{3}H-corticosterone/antibody mix was added to the appropriate number of wells of a 96-well microplate, with each sample and standard being loaded in duplicate. 20µl of either sample of standard was added to each well, the plate was covered with Parafilm and left to incubate at room temperature for 1h. 50µl of scintillation proximity assay reagent was added to each well, the wells were covered with sealant film and the samples thoroughly mixed. Samples were incubated at room temperature for 24h then counted in a Wallac 1450 Microbeta Plus scintillation

counter. Results were analysed using the Statistica software package (version 5) by ANOVA and Fisher's LSD Test, with $p<0.05$ being taken to indicate significance.

2.2.14 Sequence analysis

Sequence analysis was performed using the Wisconsin Sequence Analysis Package (GCG) (Genetics Computer Group, 1991).

3 Tissue and region-specific distribution of variant glucocorticoid receptor mRNA transcripts

3.1 Introduction

Glucocorticoid receptors are widely expressed in many tissues (section 1.4). However, levels of GR expression vary between tissues (Kalinyak et al., 1987) and even between different regions of the same tissue (Herman et al., 1989a). GR levels also vary developmentally in the hippocampus (Andrews and Matthews, 2000) and with changes in the environment of the organism e.g. during stress (section 1.9.2.4).

The GR gene is large and complex. Strahle *et al* provided the first evidence for the existence of multiple GR mRNA variants in the mouse (Strahle et al., 1992). In the rat, there are at least 11 potential alternate exons 1, at least 6 of which are expressed *in vivo* (McCormick et al., 2000). Previous work from this laboratory has shown that these exons 1 vary in their tissue and region-specific relative expression, especially in the hippocampus. Furthermore, their existence might reflect the use of alternate promoters for transcription initiation (McCormick et al., 2000). If these exons 1 also exist in the mouse, this might indicate that alternate promoter usage is likely to be of wider importance in other species and would lay the groundwork for future manipulation of the GR promoter region in transgenic animals.

The series of experiments described in this chapter were designed to further investigate the tissue and region-specific distribution of variant exons 1 in rodent tissues, which might provide a mechanism for the sensitive control of GR levels. Specifically, we wished to address the following questions:

1. To what extent is the expression of GR mRNA containing alternate exons 1 tissue-specific?

2. Is there additional region-specific expression of these variant transcripts beyond that already described in the hippocampus?

3. Is alternate GR exon 1 usage conserved in mice?

In the rat, the brain, liver and thymus were investigated due to the known distinct physiological roles of GR in these organs and our previously published data on the existence of variant exons 1 in these tissues. In the mouse a wide range of tissues were investigated to establish the existence and possible tissue-specific expression of alternate exons 1 in this species.

3.2 Experimental design

3.2.1 Semiquantitative RT-PCR

To provide a rapid and sensitive method of detection of alternate GR mRNA transcripts we adopted a RT-PCR approach.

Sequence comparison of the rat and mouse GR gene promoter region sequences revealed 91.4% identity between the sequences, with all the known alternate exons 1 conserved in the mouse together with their splice sites (Appendix A). PCR primers were designed to be complementary to the rat GR gene sequence for exon 1_1, to the rat and mouse GR gene sequences individually for exon 1_4 and to both the rat and mouse GR gene sequence for exons, 1_5, 1_6, 1_7, 1_{10} and 1_{11} (section 2.1.8.1, Figure 3.1). However, as the original primers for exons 1_1 and 1_4 led to production of multiple PCR products, a primer complementary to the sequence of a 5'-RACE clone of the rat exon 1_1 (McCormick et al., 2000) was designed and another primer complementary to both the mouse and rat GR gene sequences was designed for exon 1_4 (section 2.1.8.1). Exons 1_2 and 1_3 were not investigated as their exact positioning on the genomic GR gene sequence is not known. Furthermore, exon 1_2 was not present at detectable levels in rat liver, hippocampus or thymus (McCormick et al., 2000). Exons 1_8 and 1_9 were not analysed. They are unlikely to be present at significant levels *in vivo* under basal conditions since they were poorly represented in the 5'-RACE PCR from rat hippocampus (McCormick et al., 2000). A single 3' primer was used, complementary to the 5' end of exon 2 of the GR gene (Figure 3.1).

The design of these primers allowed PCR products generated from a reverse transcribed cDNA to be distinguished from those generated from genomic DNA contamination of the RT reaction, which would result in the formation of much larger products (>2kb). Also, with the Taq DNA polymerase used here, these

products would be less likely to be produced as the efficiency of PCR reactions is dramatically reduced if the products are greater than 2kb in size.

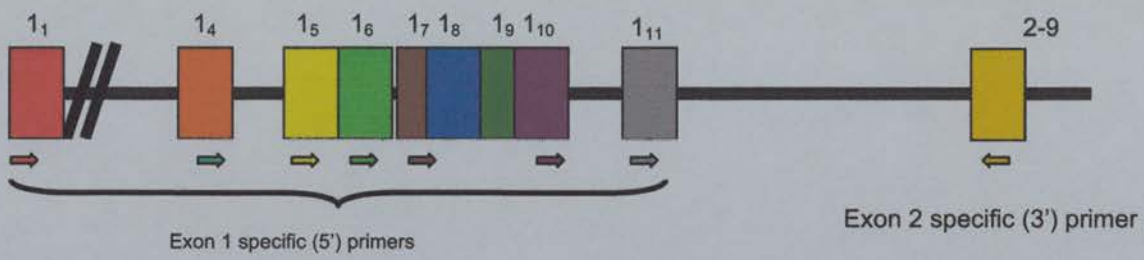


Figure 3.1: diagrammatic representation of the positions of exon 1-specific primers on the GR gene promoter region. For further details see section 2.1.8.1.

3.2.2 *In situ* mRNA hybridisation

cRNA probes used to detect GR mRNA transcripts containing exons 1_5, 1_7, 1_{10}, 1_{11} and 2 have been previously described ((McCormick et al., 2000), section 2.2.10.3) and were generated directly from PCR product templates. To be able to detect GR mRNA transcripts containing exon 1_1, a similar cRNA probe was designed (section 2.2.10.3). Briefly, extended primers containing a T7 polymerase site (3' primer) or T3 polymerase site (5' primer) were designed based on the sequences for mouse exon 1A published by Chen *et al* (Chen et al., 1999a). These primers were used to PCR amplify exon 1_1 from cDNA produced from mouse thymus total RNA as a template. Expression of GR mRNA transcripts containing exon 1_6 was not examined, despite previous data showing their presence in rat hippocampus, liver and thymus (McCormick et al., 2000). Due to the highly GC-rich nature of the DNA within and surrounding exon 1_6 (see Appendix A) it was not possible to design a specific cDNA probe with a sufficiently low annealing temperature to allow specific hybridisation. *In situ* mRNA hybridization with a riboprobe specific for exon 2 of the GR gene (common to all GR mRNA variants) was performed to reveal the distribution of the total population of GR mRNA transcripts. This probe is similar in size to the exon 1 probes (section 2.2.10.3) and was previously shown to give an identical distribution of binding (McCormick et al., 2000) to the standard GR probe complementary to exons 5-9 of the GR gene (Holmes et al., 1995b; Holmes et al., 1995a; Yau et al., 1994; Yau et al., 2001a; Yau et al., 1997b) used in our laboratory. The cRNA probe complementary to exons 5-9 was also used in some experiments to validate the data provided by the exon 2 probe.

3.3 Results

3.3.1 Variant GR mRNA transcripts are expressed in a wide range of rat tissues and vary in their tissue-specific relative expression.

RT-PCR demonstrated the presence of all exon 1 variants in rat tissues. Exons 1_5, 1_6, 1_7, 1_{10} and 1_{11} appeared to be ubiquitously present while exons 1_1 and 1_4 were not (Table 3.1, Figure 3.2 and Figure 3.3). The specificity of the observed bands was

confirmed by a comparison of the observed and expected product sizes. Negative controls without the use of reverse transcriptase produced no bands after PCR amplification (data not shown). Semiquantitative analysis showed tissue-specific differences in relative expression levels (Table 3.1). For example, transcripts containing exon 1_4 appeared relatively scarce in cerebellum and thymus but relatively abundant in hippocampus (Table 3.1). GR mRNA containing exon 1_5 was expressed at low levels in cerebellum, hippocampus and thymus but was more abundant in liver (Table 3.1, Figure 3.3). In contrast, transcripts containing exon 1_6, 1_{10} and 1_{11} were relatively abundant in all tissues examined (Table 3.1).

Tissue	Exon 1_1	Exon 1_4	Exon 1_5	Exon 1_6	Exon 1_7	Exon 1_{10}	Exon 1_{11}
Cerebral cortex	ND	ND	+	+++	+	+++	+++
Cerebellum	ND	+	+	++++	+	++++	+++
Hippocampus	++	++++	+	++++	+++	++++	++
Liver	+++	++	+++	++++	++	++++	+++
Thymus	+++	+	+	++++	+	++	++

Table 3.1: distribution and relative amounts of variant exons 1 in tissues from control rats.

Each assay was performed twice.

Key: **ND** **not detected** ++++ **present at 1/125 dilution**

+++ **present at 1/25 dilution** ++ **present at 1/5 dilution**

\+ **present only in neat RT product**

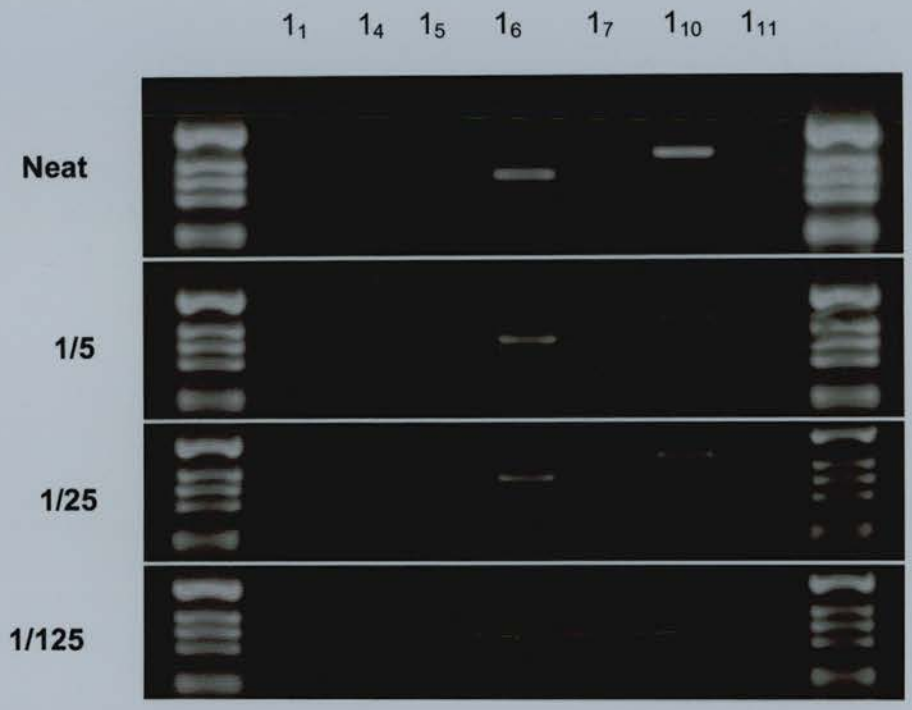


Figure 3.2: representative gel showing PCR products obtained after amplification of serial dilutions of cDNA made from rat cerebral cortex RNA.

The exon 1-specific primer used to generate the product in each lane is indicated at the top of the figure and the dilution of RT product used for each reaction is indicated at the left-hand side.

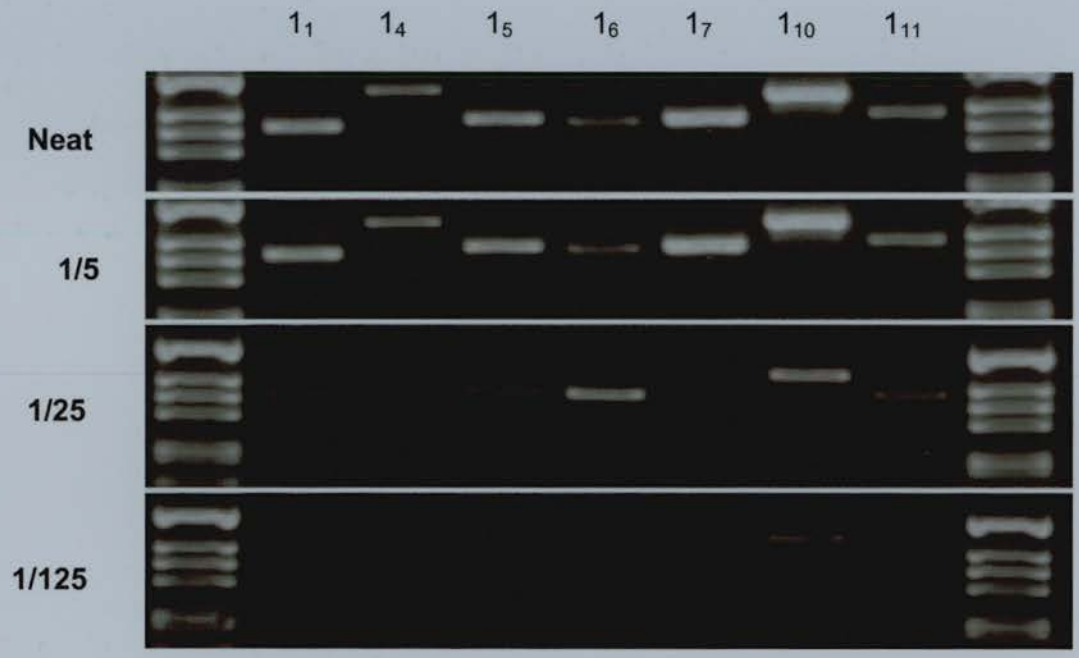


Figure 3.3: representative gel showing PCR products obtained after amplification of serial dilutions of cDNA made from rat liver RNA.

The exon 1-specific primer used to generate the product in each lane is indicated at the top of the figure and the dilution of RT product used for each reaction is indicated at the left-hand side.

3.3.2 Variant GR mRNA transcripts are conserved in the mouse and also show tissue-specific variation in expression levels.

RT-PCR showed the presence of all the predicted bands in a variety of mouse tissues, demonstrating that use of alternate exons 1 is conserved between rat and mouse (Table 3.2).

The original rat and mouse-specific exon 1_4 primers produced multiple bands in mouse PCRs. Despite designing a replacement primer complementary to both sequences, multiple bands were still obtained in mouse RT-PCRs using this primer. To determine which band was specific for exon 1_4, Southern blotting was performed on 1_4 PCR products from a selection of rat and mouse tissues with a cDNA probe specific for exon 1_4. The results indicated that the specific band was approximately 450bp in rat and 350bp in the mouse (data not shown).

The exon 1_1 primer produced a single product in mouse PCRs, but the product was larger than expected (≈800bp compared to ≈200bp). Initially, the most likely reason for this difference was thought to be a sequence difference between the rat and mouse exons 1_1. However, after the publication of the mouse exon 1A sequence (Chen et al., 1999a) it was found that the 1_1 primer was complementary to a section of sequence whose 3' end was 3 nucleotides from the 3' end of exon 1A (Chen et al., 1999a). It is possible that the amplified mouse exon 1A cDNA was reverse transcribed from a mRNA transcript spliced from a downstream splice donor site similar to those reported for the human exon 1A (Breslin et al., 2001) and thus produced a longer PCR product. However, it was also possible that the longer PCR product was due to the primers amplifying cRNA from another gene. The sequences of the exon 1_1 and exon 2 primers were compared with the mouse genome using BLAST (Altschul et al., 1997). The primers matched 36 and 20 mouse genomic sequences respectively, although none of the sequences were matched by both primers. Southern analysis of PCR products using a probe complementary to exon 1_1 or sequencing of the PCR product could resolve whether the product is indeed exon 1_1.

In the range of tissues used for semiquantitative analysis, exons 1_5, 1_6, 1_7, 1_{10} and 1_{11} appeared to be ubiquitously expressed while exons 1_1 and 1_4 did not (Table 3.2, Figure 3.4 and Figure 3.5). Exon 1_{10} was relatively abundant in all tissues examined and in most tissues could be readily detected in a 1/25 dilution of RT product (Table 3.2). In contrast, whilst ubiquitously expressed, the other exons 1 showed marked variability in their tissue-specific relative expression. Exon 1_4 is not detectable in fat, lung and heart and appears relatively scarce in kidney (Table 3.2, Figure 3.4 and Figure 3.5). Expression of exon 1_7 is relatively low in abdominal fat and heart (Table 3.2).

Tissue	Exon 1_1	Exon 1_4	Exon 1_5	Exon 1_6	Exon 1_7	Exon 1_{10}	Exon 1_{11}
Lung	ND	ND	++	++	++	+++	+++
Heart	ND	ND	+	+	+	+++	+
Kidney	ND	++	+++	++	++	+++	++++
Thymus	D	++	+++	++	++	+++	++
Spleen	++++	+	++++	++++	+++	++++	++++
Fat (abdominal)	ND	ND	+++	+++	+	+++	++++
Testis	ND	+++	++++	+++	++	+++	++++

Table 3.2: distribution and relative amounts of variant exons 1 in tissues from MF1 mice.

Key: **ND** **not detected** ++++ **present at 1/125 dilution**

+++ **present at 1/25 dilution** ++ **present at 1/5 dilution**

\+ **present only in neat RT product**

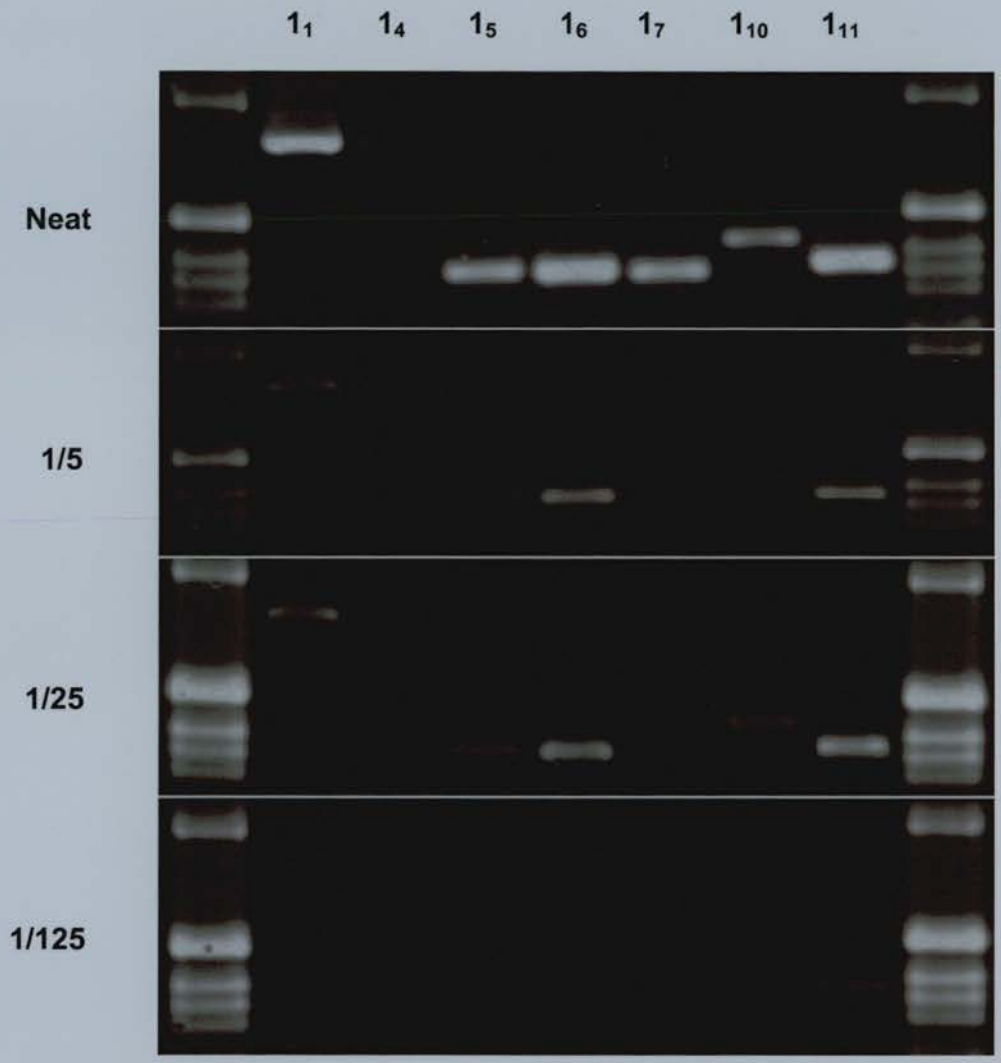


Figure 3.4: representative gel showing PCR products obtained after amplification of serial dilutions of cDNA made from mouse spleen RNA.

The exon 1-specific primer used to generate the product in each lane is indicated at the top of the figure and the dilution of RT product used for each reaction is indicated at the left-hand side.

The larger of the 2 bands in the 1_4 lane is a non-specific band with the true band of approximately 400bp only visible with the neat RT reaction (see text for details of how the specific band was identified).

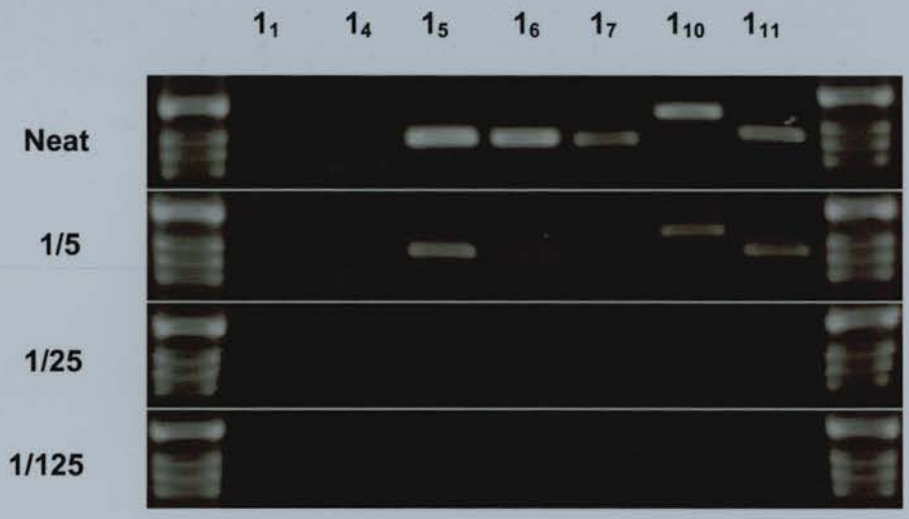


Figure 3.5: representative gel showing PCR products obtained after amplification of serial dilutions of cDNA made from mouse kidney RNA.

The exon 1-specific primer used to generate the product in each lane is indicated at the top of the figure and the dilution of RT product used for each reaction is indicated at the left-hand side.

3.3.3 Alternate exons 1 and exon 2 of the GR gene show different region-specific distributions in rat brain.

GR mRNAs containing exons 1_5, 1_6, 1_7, and 1_{10} have all been shown to be present in rat hippocampus (McCormick et al., 2000). To indicate whether these transcripts were differentially expressed within the brain, *in situ* mRNA hybridization with riboprobes specific for exons 1_5, 1_7 and 1_{10} of the GR gene was performed on serial sections obtained from the brain of a single control Wistar rat as a follow-up to the RT-PCR experiments described in section 3.3.1.

Expression of total GR mRNA (detected by the probe specific for exon 2) appears relatively uniform through the brain, with slightly higher expression in the lateral ventricle walls, septal hypothalamic nuclei, subfornical organ, paraventricular hypothalamic nucleus and cerebellar grey matter (Table 3.3, Figure 3.6). No specific hybridization was seen in the cerebellar white matter; densitometric analysis showed very similar signal with the corresponding sense control probe.

Table 3.3 (facing page): summary of mapping of expression of exon 2 and alternate exons 1 of the glucocorticoid receptor gene in the brain.

Results were derived from 156 sections derived from a single rat brain. Approximate estimation of the level of expression of each exon in different brain areas was provided by densitometric analysis, taking the average of 4-5 measurements from a single section on which each anatomical structure was clearly visible. The optical density reading was then compared to the slide background density to give a measure of relative expression in each area.

Key: **+ = weak hybridisation of probe** **++ = moderate hybridisation of probe**

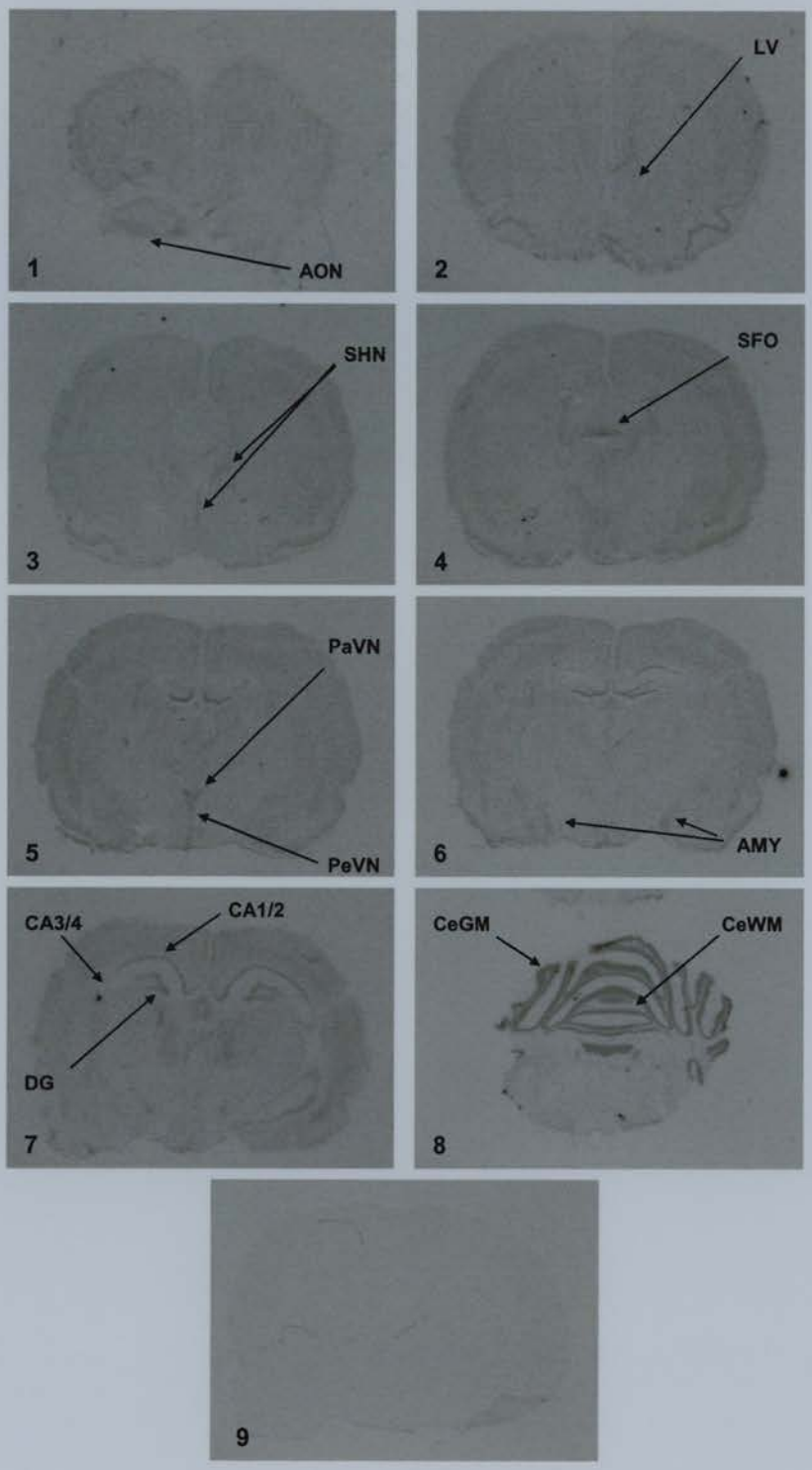
+++ = strong hybridisation of probe **++++ = very strong hybridisation of probe**

Region	Exon 2	Exon 1_5	Exon 1_7	Exon 1_{10}
Anterior olfactory nucleus	++	+	+++	++
Piriform cortex	++	++	++++	+++
Cortex layers 1-2	++	+	++++	+++
Cortex layers 3-4	++	+	+++	++
Cortex layers 5-6	++	+	+++	+++
Lateral ventricle walls	+++	+	+++	++
Preoptic nuclei	++	+	++	+++
Septal hypothalamic nuclei	+++	+	+	++++
Subfornical organ	+++	++	+++	++++
Periventricular hypothalamic nucleus	++	+	++	++
Paraventricular hypothalamic nucleus	+++	+	++	+++
Thalamic nuclei	++	++	++	++
CA1	++	++	+++	+++
CA2	++	++	+++	+++
CA3/4	+	++	++++	++
Dentate gyrus	++	+++	++++	++++
Amygdala	++	+	++	+++
Cerebellum (grey matter)	++++	++++	++++	++++
Cerebellum (white matter)	+	+	+	+

Figure 3.6 (facing page): representative autoradiographs of rat brain showing *in situ* mRNA hybridisation to a probe specific for exon 2 of the GR gene.

Panels 1-8 show binding of the antisense probe; panel 9 shows binding of the sense control probe to hippocampus.

AON = anterior olfactory nucleus, LV = walls of lateral ventricle, SHN = septal hypothalamic nuclei, SFO = subfornical organ, PaVN = paraventricular hypothalamic nuclei, PeVN = periventricular hypothalamic nuclei, AMY = amygdaloid nuclei, CA1/2 = cornu ammonis regions 1 and 2 of hippocampus, CA3/4 = cornu ammonis regions 3 and 4 of hippocampus, DG = dentate gyrus of hippocampus, CeGM = cerebellar grey matter, CeWM = cerebellar white matter.


1
AON
2
LV
3
SHN
4
SFO
5
PaVN
PeVN
6
AMY
7
CA3/4
CA1/2
DG
8
CeGM
CeWM
9

In the sections derived from the single Wistar control brain used in this experiment, all the exons 1 studied showed differences in their region-specific pattern of expression compared to the pattern of binding detected for the probe specific to exon 2 of the GR gene. Expression of exon 1_1 was studied since RT-PCR had demonstrated expression of GR mRNA containing this exon in the hippocampus (section 3.3.1). However, no specific hybridization was seen with the exon 1_1 probe. Although it bound weakly throughout the brain, similar binding was seen with the corresponding sense control probe (Figure 3.7). In contrast,G R mRNA containing exons 1_5, 1_7 and 1_{10} was expressed in the rat brain. In this brain, the distribution of GR mRNA containing exons 1_5 and 1_7 was similar. Expression of exon 1_5 was slightly higher in piriform cortex, subfornical organ, thalamus, hippocampus and cerebellar grey matter than the rest of the brain (Table 3.3, Figure 3.8). Exon 1_7 showed lower relative expression in the preoptic and septal hypothalamic nuclei, hypothalamic nuclei, thalamus, amygdala and cerebellar white matter than in the other areas studied (Table 3.3F igure 3.9). The distribution of GR mRNA containing exon 1_{10} was more homogeneous than that of exon 1_7: interestingly, exon 1_{10} was generally more highly expressed in the same areas that exon 2 was more highly expressed. Exon 1_{10} also showed the same distribution as exon 2 in hippocampus (Table 3.3, Figure 3.10).

In this brain, the distribution of the alternate GR exons 1 seemed to be particularly distinct in the hippocampus. Exon 1_{10} hybridisation mirrored the distribution of total GR mRNA, with higher expression in CA 1/2 and the dentate gyrus than in CA 3 and 4 (Figure 3.6, Figure 3.10). In contrast, hybridization of the exon 1_5 and 1_7 probes showed a more homogeneous distribution of binding through all the hippocampal subfields (Figure 3.8, Figure 3.9).

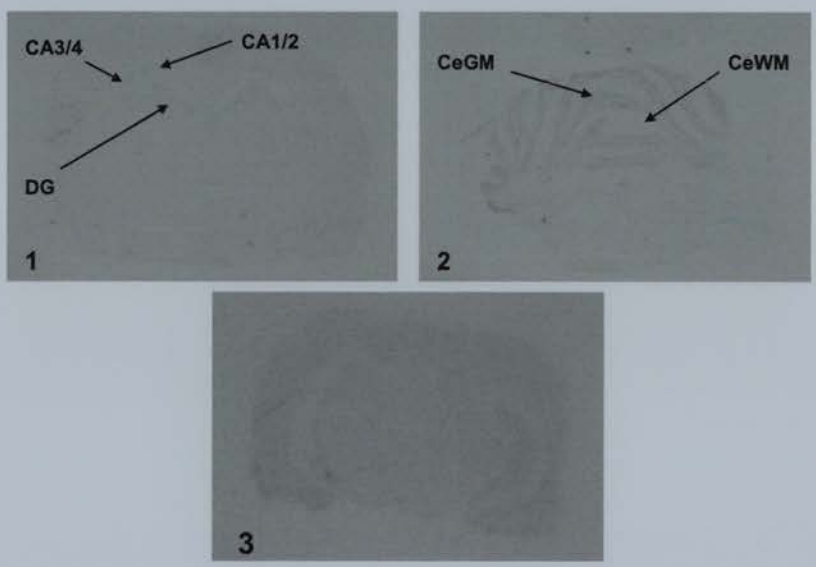


Figure 3.7: representative autoradiographs demonstrating a lack of expression of exon 1_1 of the GR gene in rat brain. Panels 1 and 2 show hybridisation of the antisense probe; panel 3 shows binding of the sense control probe to caudal hippocampus.

CA1/2 = cornu ammonis regions 1 and 2 of hippocampus, CA3/4 = cornu ammonis regions 3 and 4 of hippocampus, DG = dentate gyrus of hippocampus, CeGM = cerebellar grey matter, CeWM = cerebellar white matter.

Figure 3.8 (facing page): representative autoradiographs showing expression of GR mRNA containing exon 1_5 in rat brain.

Panels 1-8 show *in situ* mRNA hybridisation of the antisense probe; panel 9 shows binding of the sense control probe to caudal hippocampus.

AON = anterior olfactory nucleus, LV = walls of lateral ventricle, SHN = septal hypothalamic nuclei, SFO = subfornical organ, PaVN = paraventricular hypothalamic nuclei, PeVN = periventricular hypothalamic nuclei, AMY = amygdaloid nuclei, CA1/2 = cornu ammonis regions 1 and 2 of hippocampus, CA3/4 = cornu ammonis regions 3 and 4 of hippocampus, DG = dentate gyrus of hippocampus, CeGM = cerebellar grey matter, CeWM = cerebellar white matter.

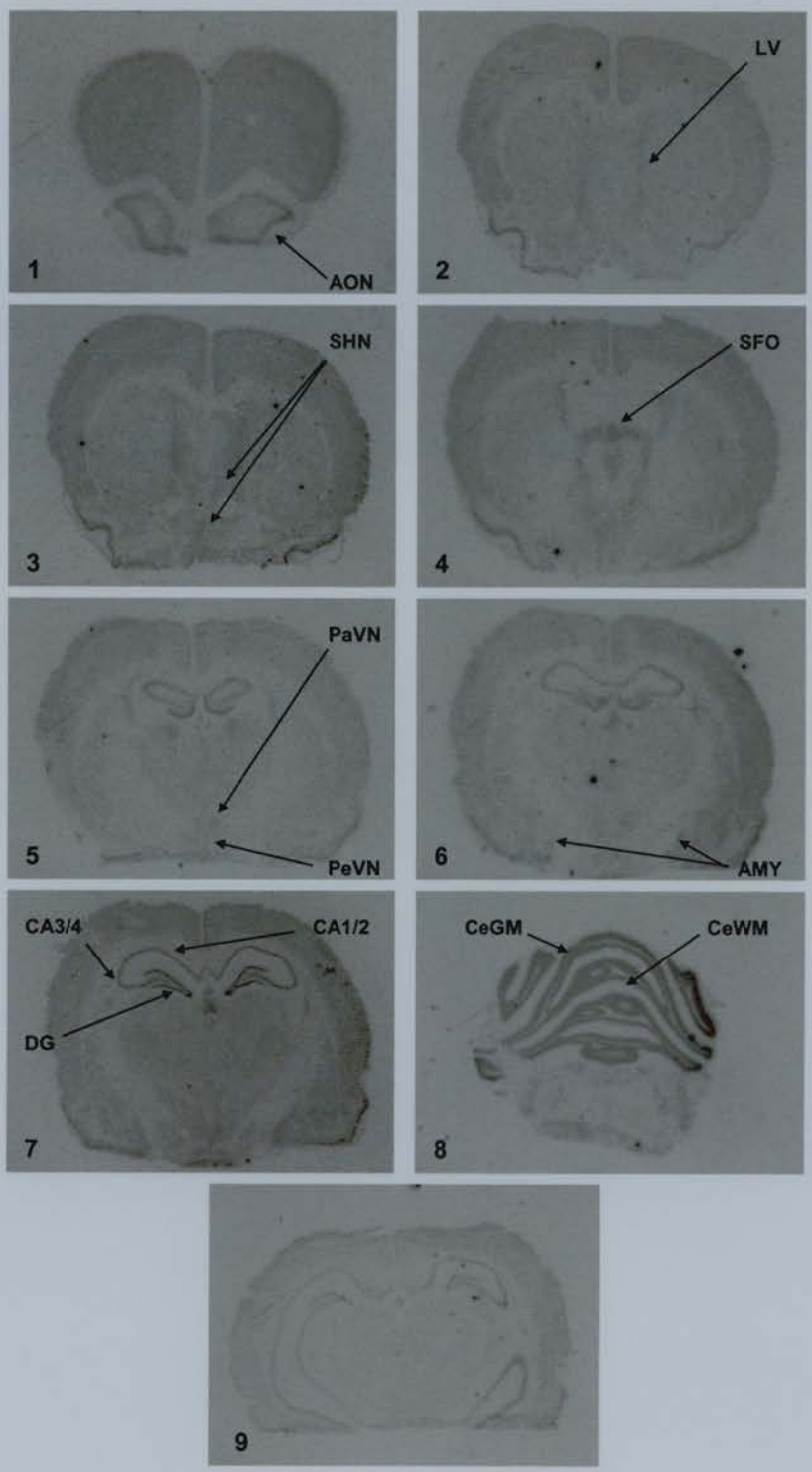
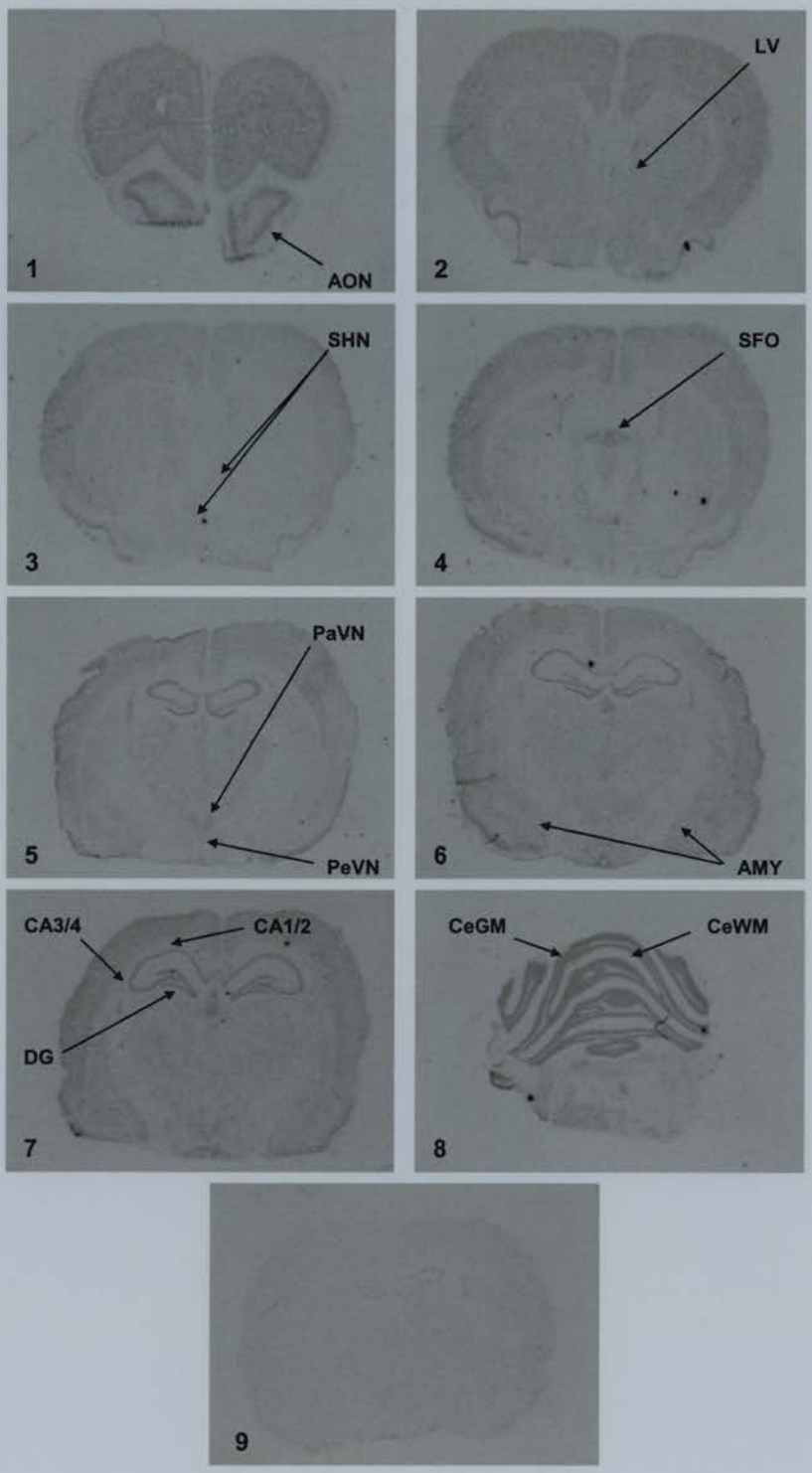

1
AON
2
LV
3
SHN
4
SFO
5
PaVN
PeVN
6
AMY
7
CA3/4
CA1/2
DG
8
CeGM
CeWM
9

Figure 3.9 (facing page): representative autoradiographs showing expression of GR mRNA containing exon 1_7 in rat brain.

Panels 1-8 show *in situ* mRNA hybridisation of the antisense probe; panel 9 shows binding of the sense control probe to cranial hippocampus.

AON = anterior olfactory nucleus, LV = walls of lateral ventricle, SHN = septal hypothalamic nuclei, SFO = subfornical organ, PaVN = paraventricular hypothalamic nuclei, PeVN = periventricular hypothalamic nuclei, AMY = amygdaloid nuclei, CA1/2 = cornu ammonis regions 1 and 2 of hippocampus, CA3/4 = cornu ammonis regions 3 and 4 of hippocampus, DG = dentate gyrus of hippocampus, CeGM = cerebellar grey matter, CeWM = cerebellar white matter.


1
AON
2
LV
3
SHN
4
SFO
5
PaVN
PeVN
6
AMY
CA3/4
CA1/2
DG
7
CeGM
CeWM
8
9

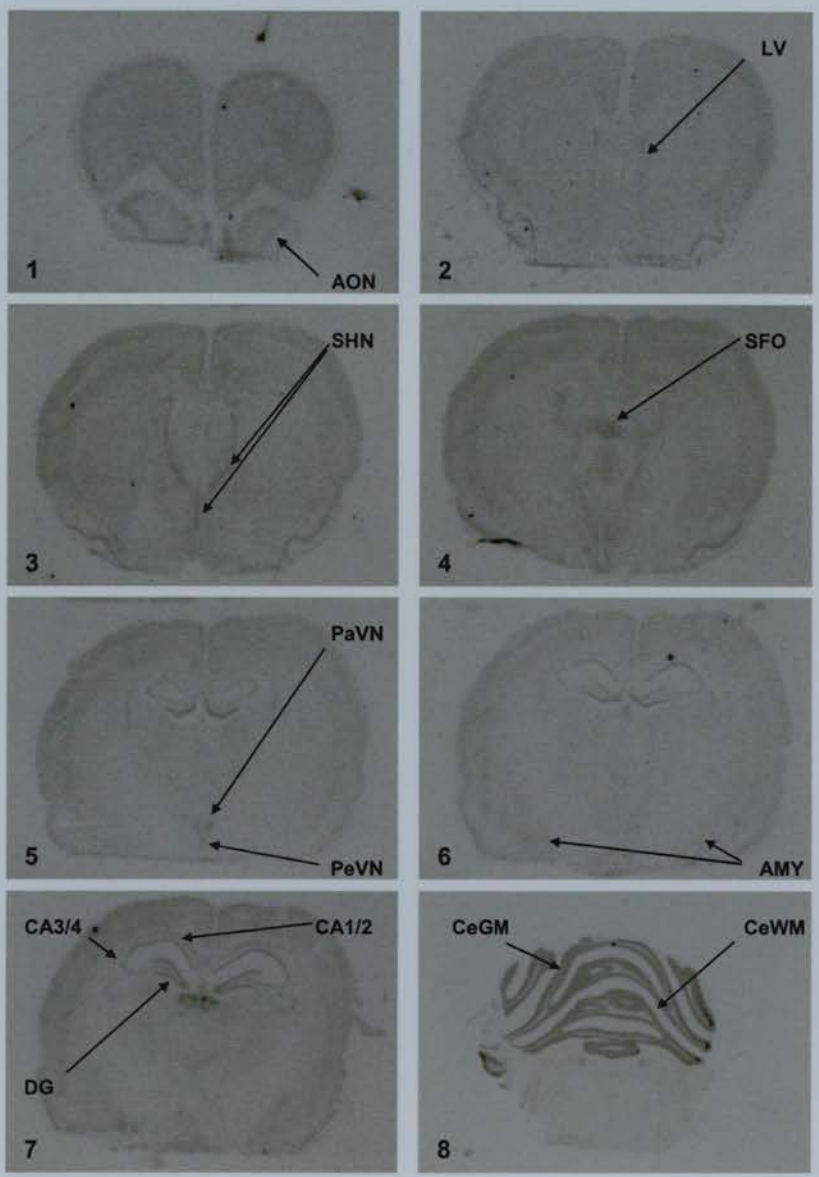


Figure 3.10: representative autoradiographs showing expression of GR mRNA containing exon 1_{10} in rat brain. No sense control probe was available for use in this study (see Section 2.2.10.3 for details). For key to labels see Figure 3.9.

3.3.4 Total GR and GR transcripts containing exon 1_{10} show a similar distribution of binding in rat liver.

Detected using a probe specific for exons 5-9, total GR mRNA shows a striking zonal distribution in rat liver (Figure 3.11). The areas expressing GR mRNA most highly are the periportal regions, while the perivenous regions show lower expression (Figure 3.11 and Figure 3.12). Transcripts containing exon 1_{10} show a similar distribution to total GR, while those containing exon 1_{11} show a more homogeneous distribution (Figure 3.11 and Figure 3.12). However, binding of the exon 1_{11} antisense probe is very similar to that of the sense probe, indicating a low level of expression of exon 1_{11} in liver.

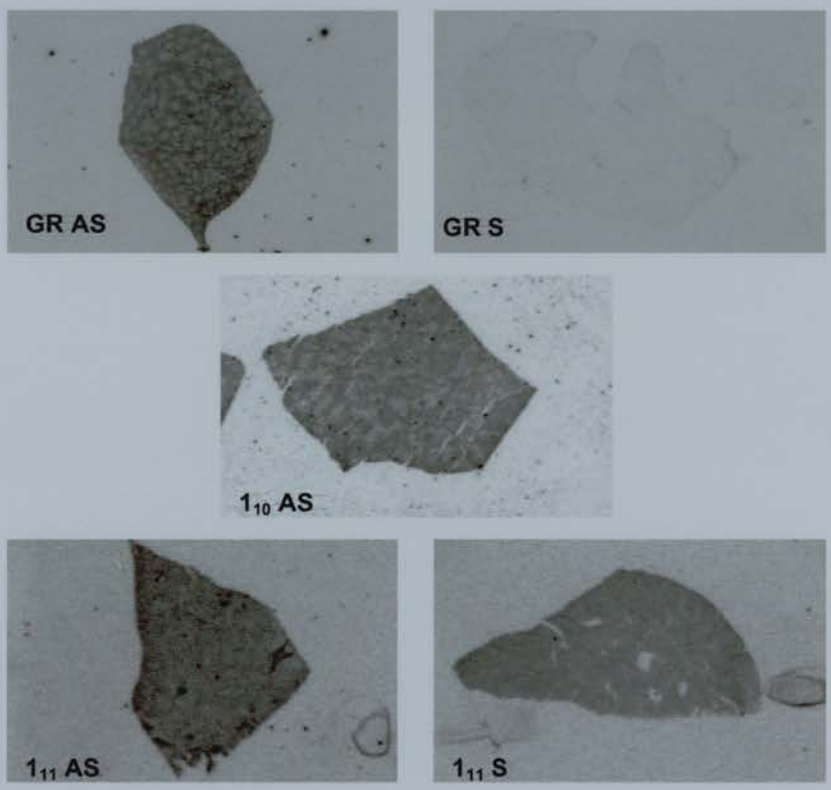


Figure 3.11: representative autoradiographs showing distribution of total GR mRNA and GR mRNA containing exons 1_{10} and 1_{11} of GR in rat liver.

Total GR mRNA and GR mRNA containing 1_{10} show a similar distribution, while exon 1_{11} shows a more homogeneous distribution (similar to that of the corresponding sense control).

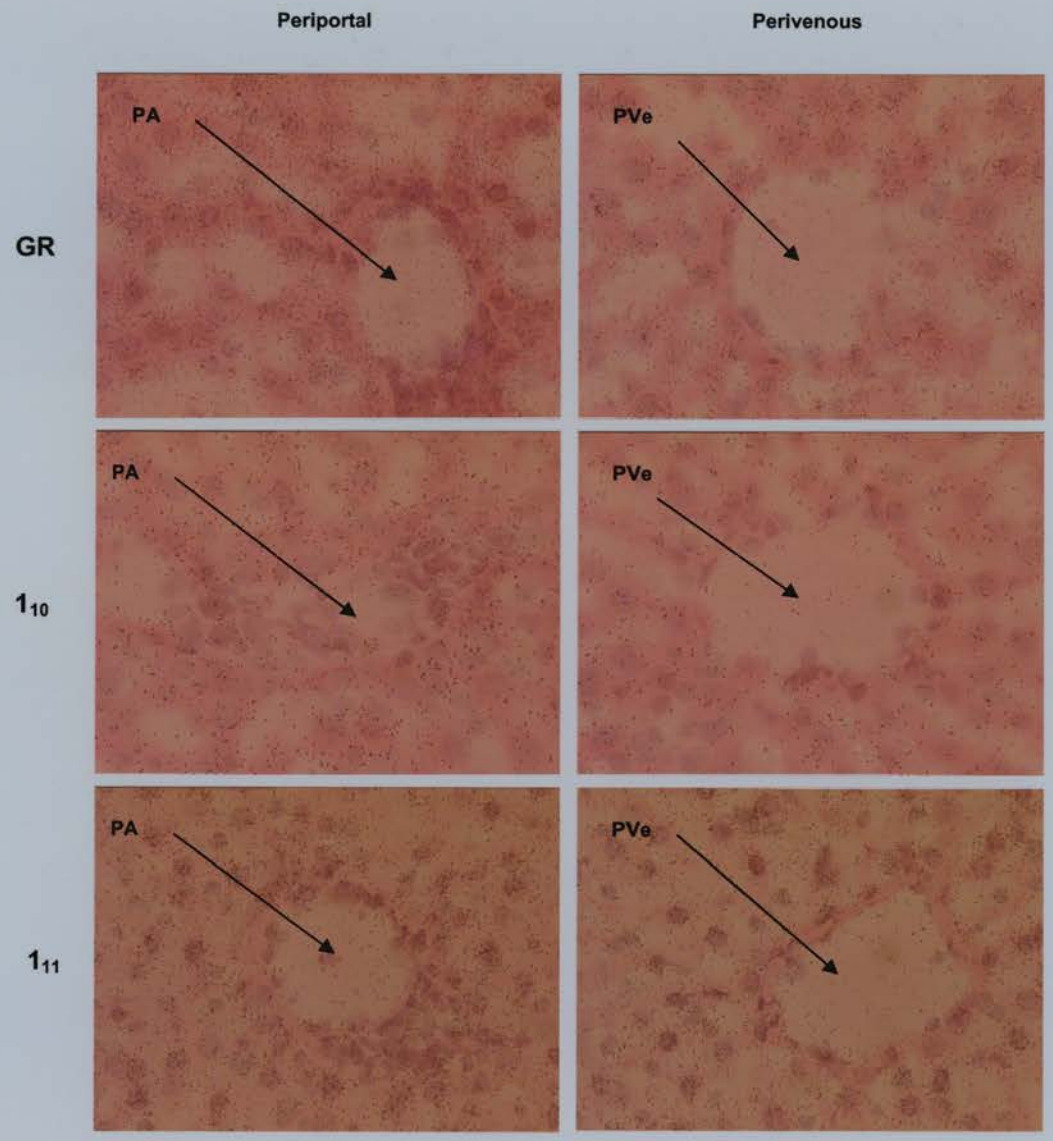
AS = antisense probe, S = sense probe.

Figure 3.12 (facing page): representative photomicrographs showing *in situ* mRNA hybridisation to fresh frozen sections of the periportal and perivenous areas of rat liver.

Note higher density of silver grains in the periportal area compared to the perivenous area for GR mRNA and GR mRNA containing exon 1_{10}, while GR mRNA containing exon 1_{11} shows a more homogeneous distribution.

Magnification x 400.

GR = exon 5-9 probe, 1_{10} = exon 1_{10} probe, 1_{11} = exon 1_{11} probe, PA = portal arteriole, PVe = portal venule.


Periportal
Perivenous
GR
PA
PVe
1_{10}
PA
PVe
1_{11}
PA
PVe

3.3.5 Total GR and GR transcripts containing alternate exons 1 are distributed similarly in rat and mouse thymus

To establish conditions for *in situ* mRNA hybridisation on thymus, two methods of fixing fresh frozen sections of rat thymus were tested; with and without partial acetylation by immersion in a solution of acetic anhydride (section 2.2.10.4). A preliminary experiment revealed that the acetylation step gave much better results with an exon 2-specific cRNA probe (Figure 3.13), so this method of fixation was used for all subsequent experiments.

Total GR mRNA was detected using the exon 2 probe. Expression of exons 1_1, and 1_{10} was also examined. Expression of other exons 1 was not examined as they were not previously detected in thymus by RNase protection analysis (McCormick et al., 2000). All probes showed greater binding in the thymic cortex than in the thymic medulla (Figure 3.14 and Figure 3.15).

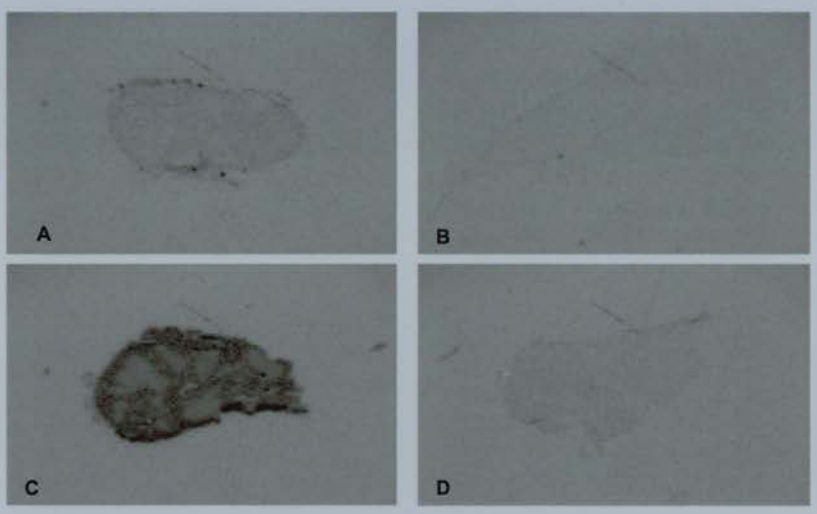


Figure 3.13: representative autoradiographs showing *in situ* mRNA hybridisation of an exon 2-specific probe to sections of rat thymus fixed with (C, D) and without (A, B) acetylation step.

Sections A and C were hybridised to the antisense probe, while sections B and D were hybridised to the sense probe. Sections were exposed to the same piece of autoradiography film for the same length of time (5d).

Note the better resolution of thymic cortex and medulla and greater difference between the hybridisation of the sense and antisense probes in the sections fixed with acetylation.

Images are taken from a 7d exposure

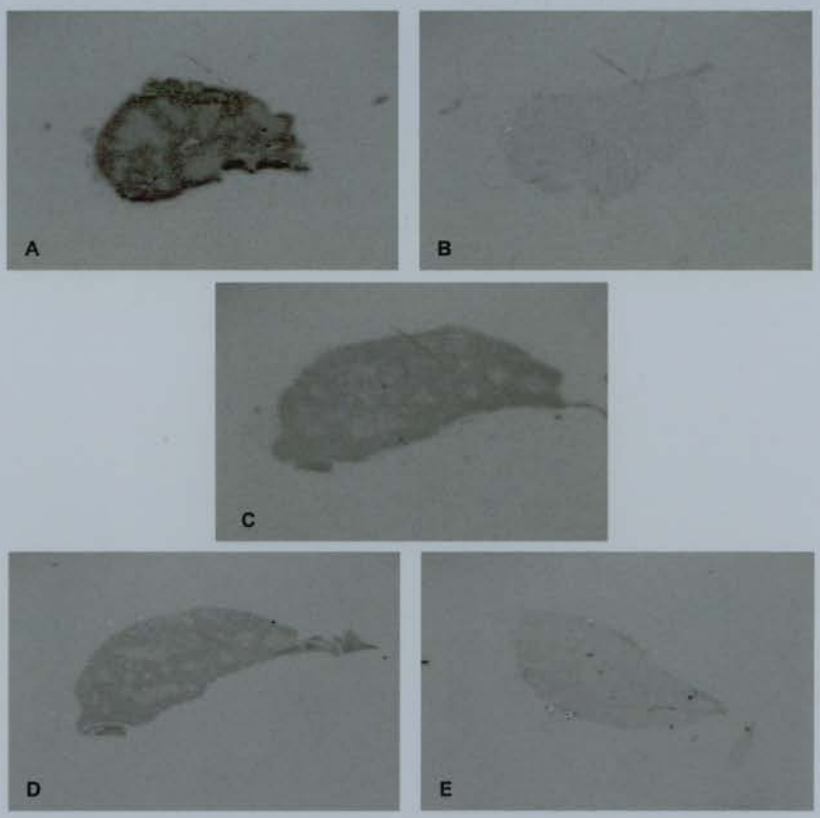


Figure 3.14: representative autoradiographs showing *in situ* mRNA hybridisation of probes complementary to exons 2, 1_{10} and 1_1 of the GR gene to rat thymus.

Panel A = exon 2 antisense probe, B = exon 2 sense probe, C = exon 1_{10} antisense probe, D = exon 1_1 antisense probe, E = exon 1_1 sense probe.

Note the similar distribution of binding for all the antisense probes. Images are taken from a 7d exposure.

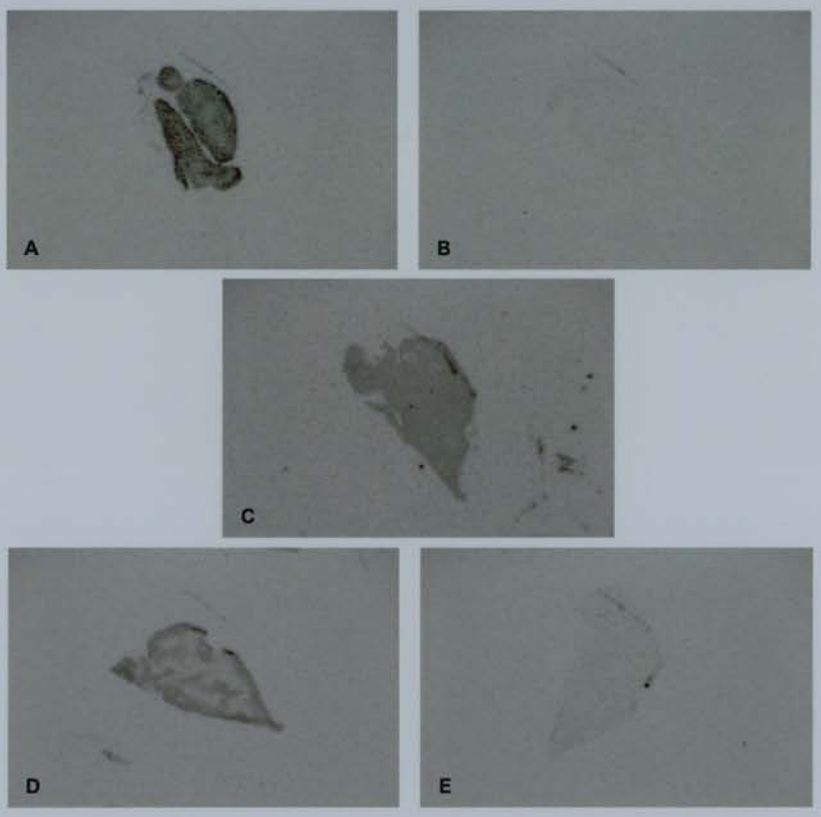


Figure 3.15: representative autoradiographs showing *in situ* mRNA hybridisation of probes complementary to exons 2, 1_{10} and 1_1 of the GR gene to mouse thymus.

Note the similar distribution of binding for all the antisense probes.

A = exon 2 antisense probe, B = exon 2 sense probe, C= exon 1_{10} antisense probe, D = exon 1_1 antisense probe, E = = exon 1_1 sense probe

3.4 Discussion

The experiments described in this chapter were designed to further investigate whether the previously-identified variant exons 1 of the rat GR gene (McCormick et al., 2000) were conserved in the mouse and whether they were differentially expressed between and within tissues.

The sequences of all the exons 1 studied that were previously identified in the rat were conserved in the mouse GR promoter genomic DNA and were identified in mouse tissues by RT-PCR. Exons 1A (equivalent to rat exon 1_1), 1B (equivalent to rat exon 1_6) and 1C (equivalent to rat exon 1_{10}) had been previously identified in a limited number of tissues and cell lines (Strahle et al., 1992). Also, exons 1D (equivalent to rat exon 1_5) and 1E (equivalent to rat exon 1_{11}) have recently been shown to be present in S-49 mouse T-lymphoma cells (Chen et al., 1999b). However, this is the first time that expression of exons 1_4 and 1_7 has been reported in the mouse.

In both the rat and mouse, most of the variant exons 1 studied were ubiquitously expressed and showed tissue-specific differences in their relative expression. It should be noted that the results of separate RT-PCR experiments from different tissues are not directly comparable. However, comparisons of the relative level of expression of each exon with those of the others within the same tissue may be made. The RT-PCR data from rat tissues are largely consistent with previous studies. McCormick *et al* used RNase protection analysis to identify GR mRNA containing exons 1_1, 1_5, 1_6, 1_7, 1_{10} and 1_{11} in rat tissues and demonstrated tissue-specific differences in their relative expression (McCormick et al., 2000). Furthermore, exon 1_6 is present in the published rat GR cDNA sequence (Miesfeld et al., 1986) and together with exon 1_{10} was identified by 5'-RACE PCR in rat liver GR mRNA (Gearing et al., 1993). Two other putative exon 1 sequences were identified in the same study, but in fact one of them corresponds to genomic sequence immediately upstream of exon 2 while the other lies within exon $1_{4.5}$ (V.Lyons and K.Chapman,

unpublished data). Interestingly, those transcripts displaying the greatest differences in relative expression between tissues (1_4 and 1_5) are those that appear less abundant when examined by RNase protection analysis (forming less than 1% and up to 8.1% of total GR mRNA transcripts respectively (McCormick et al., 2000)). In contrast, the exons displaying less variability in relative expression here tend to be those which are more abundant (McCormick et al., 2000). An exception to this is exon 1_{11}, which has relatively low abundance (forming approximately 12% of the total GR mRNA population in the hippocampus and approximately 2% in the liver (McCormick et al., 2000)) yet shows little variability in its relative expression.

Interestingly, GR mRNA transcripts containing exon 1_7 were clearly detectable in all the tissues studied despite previous data from RNase protection analysis suggesting that it was hippocampus-specific (McCormick et al., 2000). In contrast to the other exons 1, exons 1_1 and 1_4 were definitely not detectable in some tissues by RT-PCR but were easily detectable in others. Although Chen *et al* demonstrated expression of GR mRNA transcripts containing exon 1_1 in all the tissues they studied, with high levels of expression in liver and muscle (Chen et al., 1999b), the balance of evidence from other studies is that expression of exon 1_1 is restricted to the immune system (McCormick et al., 2000) and is specific to T lymphocytes (Strahle et al., 1992). In the experiments described here the greater sensitivity of RT-PCR (compared to RNase protection analysis) may have allowed the detection of extremely small numbers of transcripts containing exon 1_1 in immune cells contaminating the tissues. These could be in blood or in the tissues themselves, such as the Kupffer cells of the liver. In future experiments the possibility of blood contamination could be reduced by saline-perfusion of the animals before harvesting of tissues for RT-PCR.

Some exons 1 were readily detected by RT-PCR in tissues where RNase protection analysis failed to detect their presence (exon 1_4 in hippocampus, liver and thymus, exon 1_5 in liver and thymus, exon 1_7 in liver and thymus and exon 1_{11} in thymus) (McCormick et al., 2000). These data suggest that, in the tissues where they are detectable by RT-PCR but not by RNase protection analysis, the transcripts are either present at a low level (1-2% of the total GR mRNA) in all cells or are present at higher levels in a subpopulation of cells.

Although the tissues used in these experiments were derived from a single control animal (and hence the results should be regarded as an indication of expression rather than a quantitative measure and should be interpreted with caution, particularly with regard to whether they are representative of the population at large), it appears that there may be qualitatively different expression of the variant exons 1 studied within the rat brain. *In situ* mRNA hybridisation did not detect exon 1_1 in any of the regions studied. Thus the RT-PCR product demonstrated in hippocampal RNA is likely to be due to blood contamination. Exon 1_5 was largely absent also, apart from in the piriform cortex, subfornical organ, hippocampus and cerebellum. Interestingly, RT-PCR also demonstrated relatively low expression of transcripts containing exon 1_5 compared to other GR mRNA transcripts in cerebellum, cerebral cortex and hippocampus. These observations are in agreement with previous data showing that exon 1_5 comprised ≈8% of GR mRNA in rat hippocampus and showed a homogeneous distribution throughout this structure (McCormick et al., 2000). GR mRNA transcripts containing exon 1_7 were detected by *in situ* mRNA hybridisation in all brain areas, in agreement with the RT-PCR data, and were distributed in hippocampus as reported previously (McCormick et al., 2000). GR mRNA transcripts containing exon 1_{10} have been shown to be similarly distributed to total GR mRNA in hippocampus (McCormick et al., 2000): this study shows that this is likely to be the case throughout the brain, probably reflecting their predominance in the total GR mRNA population in all tissues studied so far (McCormick et al., 2000). Total GR mRNA was expressed in all brain areas studied with a distribution similar/identical to that reported previously (Herman et al., 1989a; Sousa et al., 1989). Minor differences in results are likely to be due to specificity of probes (both the above studies used considerably longer riboprobes of 1155 and 452 nucleotides respectively that were targeted towards the 3' end of the GR gene) or subjective assessment of the relative GR mRNA expression. Also, since all the tissues used in this experiment were derived from a single brain, it is possible that there is some individual variation in the distribution of total GR mRNA and variant exons 1. Further experiments would be required to determine if this is the case.

The differences in the pattern of distribution of the different exons 1 and total GR mRNA suggests that the different exons 1 form different proportions of the total GR mRNA population in different brain areas, again suggesting there is region-specific differential expression of exon 1 variants in brain. The high level of hybridisation of all the antisense probes seen in the cerebellar grey matter was most probably due to the extremely high density of cells in the granular cell layer which can also cause higher hybridisation of the sense probe (Sousa et al., 1989).

In the liver, GR mRNA containing exon 1_{11} does not appear to be highly expressed and shows no region-specific distribution. This data is consistent with RNase protection analysis that found exon 1_{11} present in only 2% of total GR mRNA in liver (McCormick et al., 2000). As in the hippocampus, transcripts containing exon 1_{10} have a similar distribution to total GR mRNA, which is strongly expressed in the liver with higher expression periportally as shown previously (Nyirenda et al., 1998). The relevance of this striking zonal distribution of GR may be that the distribution of a number of metabolic functions (carbohydrate metabolism, lipid metabolism, xenobiotic metabolism and amino acid metabolism) and the enzymes involved in them varies between the periportal and perivenous hepatocytes (Jungermann and Kietzmann, 1996). Enzymes and proteins involved in gluconeogenesis, urea formation, cholesterol synthesis, oxidative metabolism and bile formation are expressed mainly in the periportal region, while those involved in glucose uptake, glutamine formation and xenobiotic metabolism occur mainly in the perivenous region. The expression of several enzymes that are more highly expressed periportally is regulated by glucocorticoids. Activity of phosphoenolpyruvate carboxykinase (PEPCK) is higher periportally (Jones and Titheradge, 1996) and expression of PEPCK is upregulated by glucocorticoids (Brien et al., 1990; Sasaki et al., 1984). The expression of glucose-6-phosphatase is also upregulated by glucocorticoids (Argaud et al., 1996). Expression of fructose-2,6-bisphosphatase is under indirect control by glucocorticoids via glucocorticoid regulation of levels of fructose-2,6-bisphosphatase (Marker et al., 1989), which catalyses its synthesis and degradation (Jungermann and Kietzmann, 1996). Expression of tyrosine aminotransferase (Ganss et al., 1994) and carbamoyl phosphate synthetase (Gautier

et al., 1977; Nebes and Morris, 1988) is also upregulated by glucocorticoids, while expression of HMG CoA reductase is upregulated by glucocorticoids in cultured cells (Cavenee and Melnykovych, 1977). Thus it seems plausible that the reason for a higher level of GR in the periportal region is that it will confer greater sensitivity on the glucocorticoid control of these metabolic processes.

In the thymus, total GR mRNA was more abundant in thymic cortex than medulla, consistent with maturing thymocytes migrating to the medulla (Sprent et al., 1988) where their GR expression is reduced to a low level similar to that found in peripheral blood lymphocytes (Ranelletti et al., 1987). GR mRNA transcripts containing exons 1_1 and 1_{10} were also more abundant in thymic cortex than medulla. Transcripts containing exon 1_1 form approximately 20% of the total GR mRNA in thymus total RNA (McCormick et al., 2000) and RNA isolated from thymocytes, thymic epithelium and spleen (Dammerman A., unpublished observations). Transcripts containing exon 1_{10} are reported to form ≈50% of the total GR mRNA in thymus ((McCormick et al., 2000) and Dammerman A., unpublished observations). Thus we would expect to find higher levels of mRNAs containing these alternate exons 1 in the immature thymocytes of the cortex than in the mature cells in the medulla, in line with the level of total GR mRNA. The density of cells in the thymic medulla might be lower than in the cortex (Chapter 6), which could at least partly account for the lower density of autoradiographic signal in the medulla. Also, there could be developmental regulation of variant exons 1 e.g. exon 1_1 could be downregulated during thymocyte maturation, accounting for the reduction in total GR expression.

The tissue and region-specific variations in their relative expression suggest that the mechanisms that contribute to differential expression of variant GR mRNAs are not absolute, but rather apply to a greater or lesser extent in all tissues (with the possible exceptions of exons 1_1 and 1_4). In the case of exon 1_1, it is plausible that its position on the genomic DNA at least 15kb 5' of the CpG island containing the other exons 1 means that its associated promoter is controlled differently to those associated with the other exons 1. In addition, although exon 1_4 is located in the CpG island it lies

towards the 5' end, so it is possible that its promoter also behaves differently from those of the exons 1 lying further 3'.

The conservation of these exon 1 variants in rat and mouse and recent work demonstrating the presence of human equivalents of some (Breslin et al., 2001) or all (Reynolds, R, unpublished observations) of the alternate exons 1 studied here suggests that they are likely to be of physiological relevance. The tissue-specificity of exon 1_1 and 1_4 expression, the evidence demonstrating that exon 1_7 shows its highest associated promoter activity in CNS-derived cells (McCormick et al., 2000) and the changes in relative expression of exon 1_7 after neonatal handling and exon 1_{10} after prenatal dexamethasone exposure (McCormick et al., 2000) further support this hypothesis.

These data are consistent with variant exons 1 of the GR gene reflecting the tissue-specific use of alternate promoters, which led to the hypothesis that changes in alternate promoter usage (and hence exon 1 expression) might provide a mechanism for tissue-specific regulation of GR levels. One of the main regulators of GR levels, at least in adult animals, is levels of glucocorticoids themselves. The investigation of regulation of these alternate exons 1 in brain, liver and thymus by manipulations of glucocorticoid levels is described in the following chapters.

4 The effect of glucocorticoid manipulation on the expression of alternate exons 1 of the GR gene in the rat hippocampus

4.1 Introduction

The hippocampus contains the highest concentration of GR in the brain (section 1.9.2). Levels of GR in the hippocampus can be permanently "programmed" by early-life events. Hippocampal GR levels are increased by neonatal handling (O'Donnell et al., 1994), associated with increased expression of GR mRNA containing exon 1_7 in hippocampus but not of that containing exon 1_{10} (McCormick et al., 2000). Similar programming effects are seen in the liver after prenatal treatment with dexamethasone, where an increase in hepatic GR levels is associated with a decrease in the proportion of the total GR mRNA population containing exon 1_{10} (McCormick et al., 2000), implying an increase in expression of mRNAs encoding another exon 1 variant. GR levels are also dynamically regulated by glucocorticoids in a region and tissue-specific manner (section 1.7.2.1). The data from programmed animals (McCormick et al., 2000) led to the hypothesis that glucocorticoid-induced changes in alternate promoter usage (and consequently in expression of GR mRNAs containing different exons 1) was the mechanism by which tissue-specific glucocorticoid autoregulation of GR levels occurred.

The experiments described in this chapter were designed to test this hypothesis by examining glucocorticoid regulation of variant exon 1 expression in the hippocampus. The hippocampus was chosen (rather than other brain areas involved in control of HPA axis activity) for several reasons. Most importantly, glucocorticoid regulation of hippocampal GR levels had been previously demonstrated and was well-characterised (section 1.7.2.1). Also, most variant exons 1 are expressed in the hippocampus (chapter 3 and (McCormick et al., 2000)) and differential regulation of variant exons 1 in hippocampus by neonatal handling had already been demonstrated (McCormick et al., 2000). Finally, the hippocampus contains a high concentration of GR mRNA (Herman et al., 1989a; Sousa et al., 1989) and hence relatively abundant expression of variant exons 1. Since transcripts containing each exon 1 form only a

proportion of the total population of GR mRNA, this would facilitate the detection of individual exon 1 variants.

4.2 Experimental design

4.2.1 Adrenalectomy and glucocorticoid replacement

The effects of glucocorticoids on variant exon 1 expression were examined over two experimental periods. Short-term adrenalectomised animals (ST) underwent glucocorticoid manipulations over a period of 72h. This period was chosen because previous work from this laboratory had shown significant changes in hippocampal glucocorticoid receptor mRNA levels 2 days after adrenalectomy (Holmes et al., 1995b). Long-term adrenalectomised animals (LT) underwent glucocorticoid manipulations over a period of 3 weeks to determine the effects of these manipulations on variant exon 1 expression over a longer time period. For details of experimental procedures and animal groups see section 2.2.1.

4.2.2 *In situ* mRNA hybridisation

This technique was used for analysis of mRNA containing exon 1 variants. Although RNase protection analysis would have allowed more precise quantitation of mRNAs, it does not provide any information on the anatomical distribution of the transcripts, which is important in a heterogeneous structure like the hippocampus. Total GR mRNA levels were also assessed by *in situ* mRNA hybridisation to determine if glucocorticoid manipulations had affected hippocampal GR expression.

4.3 Results

4.3.1 Experimental manipulations significantly alter corticosterone levels in ST and LT animals.

In the ST animals, all the experimental groups showed at least an 8-fold reduction in their plasma corticosterone levels compared to the sham-operated controls (Figure 4.1 A). In this analysis (Figure 4.1 A), Adx animals had significantly lower corticosterone levels than Adx/supra animals. Despite a relatively large difference in the mean levels for the Adx and Adx/phys groups (0.78nM and 42nM respectively) the difference failed to reach statistical significance.

In the LT animals, Adx animals had significantly lower corticosterone levels than sham-operated controls, while Adx/phys animals showed no difference in corticosterone levels from sham-operated controls (Figure 4.1 B).

Figure 4.1 (facing page): radioimmunoassay of plasma corticosterone levels in trunk blood.

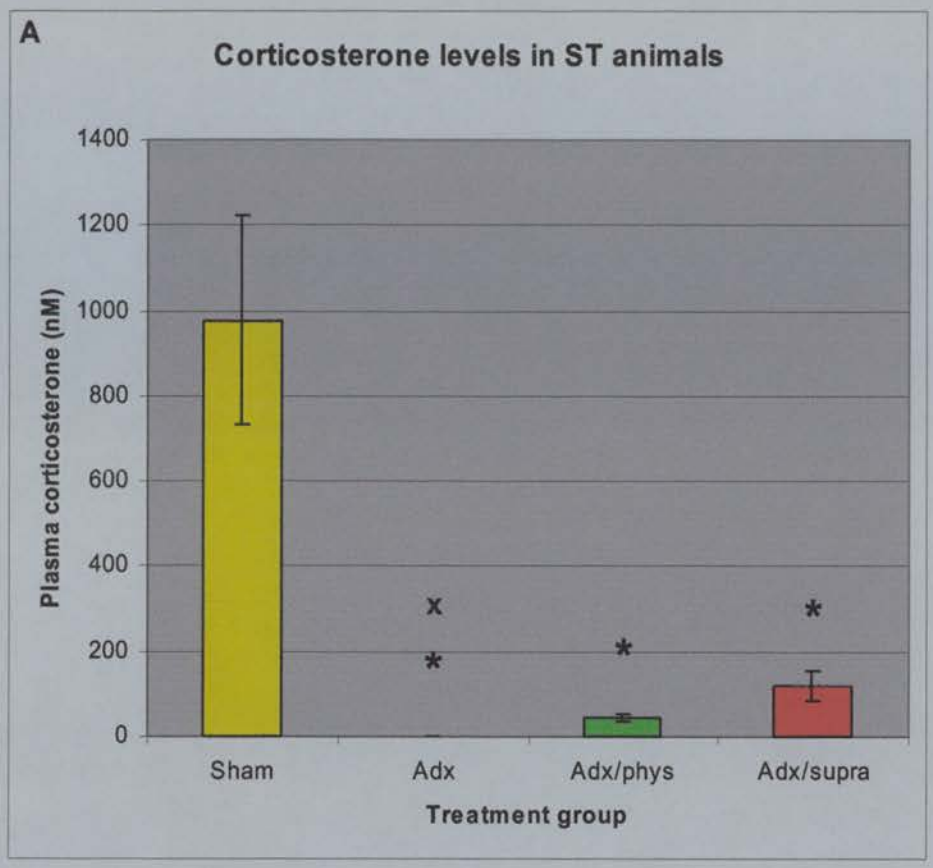
Blood samples were taken 17h after the final injection of corticosterone acetate. Data (presented as mean±SEM) were analysed by ANOVA and Tukey's HSD test.

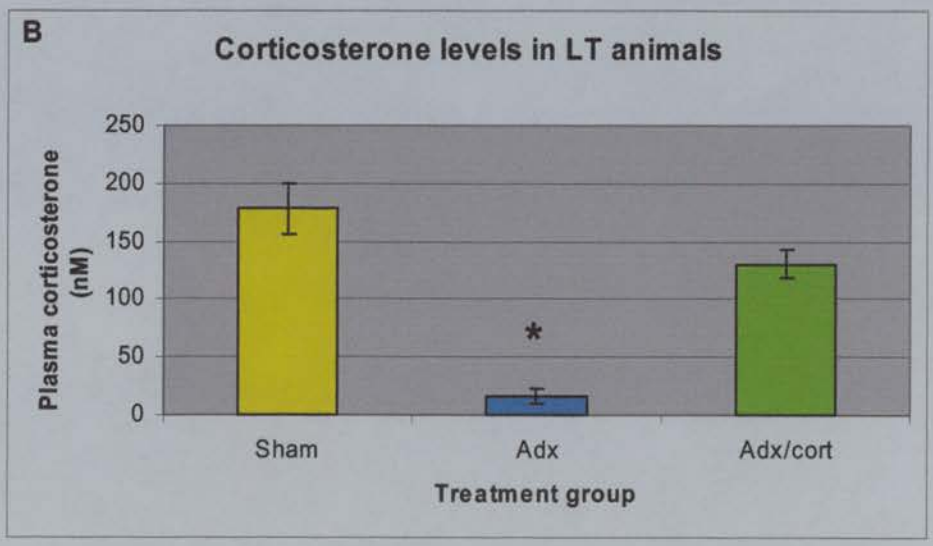
A: ST animals. As the corticosterone levels for the sham group were so much higher than those for the experimental groups, the data for the experimental groups were analysed separately.There was a significant effect of treatment ($p<0.05$), with all other groups showing a significantly lower corticosterone concentration than sham-operated controls ($p<0.05$, indicated by *). Additionally, Adx animals had significantly lower corticosterone levels than Adx/supra animals ($p<0.05$, indicated by x).

B: LT animals. There was a significant effect of treatment, with Adx animals having a lower plasma corticosterone concentration than sham-operated controls and Adx/cort animals ($p<0.05$, indicated by *).

ST = short-term adrenalectomised. LT = long-term adrenalectomised.

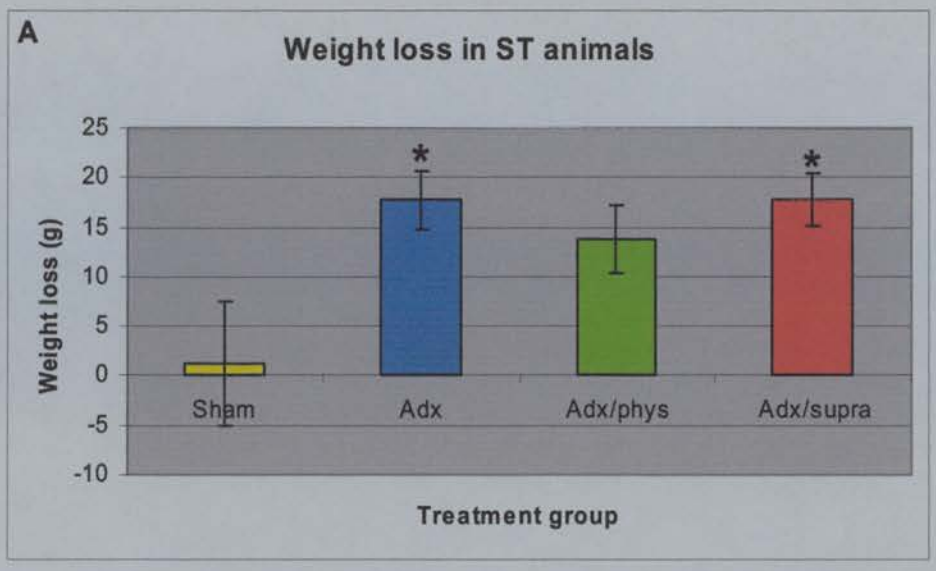
ST n=6 per group, except sham n=8. LT n=8 except Adx n=7.


A
Corticosterone levels in ST animals
Plasma corticosterone (nM)
1400
1200
1000
800
600
400
200
0
Sham
Adx
Adx/phys
Adx/supra
Treatment group


B
Corticosterone levels in LT animals
Plasma corticosterone (nM)
250
200
150
100
50
0
Sham
Adx
Adx/cort
Treatment group

4.3.2 Adrenalectomised animals and animals adrenalectomised with supraphysiological glucocorticoid replacement lose significantly more weight than sham-operated controls over the experimental period.

Both the ST and LT animals lost weight over the course of the experiments. However, the groups differed in the amount of weight lost. In the ST animals, all the treatment groups tended to lose more weight than the controls although when analysed using Tukey's HSD Test the differences between individual groups did not reach statistical significance. However, analysis using Fisher's LSD test showed that the Adx and Adx/phys animals lost significantly more weight than sham-operated controls (Figure 4.2 A). In the LT experiment, the Adx animals lost significantly more weight than sham-operated controls (Figure 4.2 B).



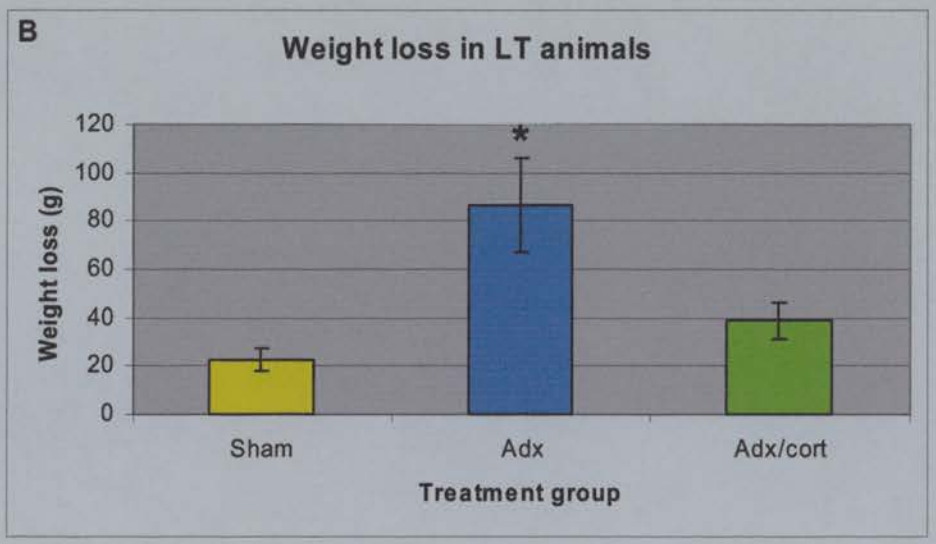


Figure 4.2: weight loss of rats over the experimental period.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

A: ST animals. There was a significant effect of treatment ($p<0.05$). Post-hoc analysis using Tukey's HSD Test failed to reveal significant differences between individual groups, but Fisher's LSD Test showed that Adx and Adx/supra animals lost significantly more weight than sham-operated controls ($p<0.05$, indicated by *). n=6 except sham n=8.

B: LT animals. There was a significant effect of treatment, with Adx animals losing significantly more weight than sham-operated controls ($p<0.05$, indicated by *). n=8 except Adx n=7.

4.3.3 RNA probes generated from the GR exon 2 template in different vectors give qualitatively different results in *in situ* mRNA hybridisation.

The *in situ* mRNA hybridisation experiments described in Chapter 3 were performed using probes generated directly from PCR product templates. However, it was noted that the yield (measured in disintegrations/second/µl of eluted probe) of RNA probe obtained by *in vitro* transcription using these templates was quite variable and was frequently low. In order to increase the reliability and yield of probe synthesis, the PCR products were subcloned into pGEM-T Easy as described in section 2.2.10.3. However, the probes produced from the constructs utilising the pGEM-T Easy vector system gave variable results. In particular, the exon 2 cRNA probe synthesised using the subcloned PCR product as template gave a different distribution of binding in hippocampus to that of the probe synthesised directly from the PCR product template (Figure 4.3). This distribution did not agree with the previously published distribution of GR in hippocampus (Herman et al., 1989a). On further investigation, it was discovered that if the transcribed antisense probe contained the region of the pGEM-T Easy polylinker sequence between the T7 polymerase recognition site and the insert, it gave an abnormal distribution of antisense binding. If this section of polylinker was present in the sense probe, it caused abnormally high binding of the sense probe (data not shown). To eliminate this possible source of error for exon 1 probes, the PCR product templates for the variant exons 1 were recloned as described in section 2.2.10.3, which resolved the problem. *In situ* mRNA hybridisation experiments to detect total GR mRNA were subsequently performed using a probe complementary to exons 5-9 of the GR gene.

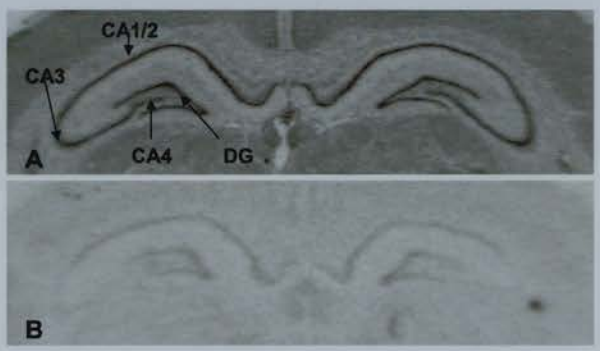


Figure 4.3: representative autoradiographs showing *in situ* mRNA hybridisation of probes specific for exon 2 of the GR gene to rat hippocampus.

A: hybridisation of a probe transcribed from the exon 2 PCR product subcloned into pGEM-T Easy. 21d exposure.

B: hybridisation of a probe transcribed directly from the PCR product. 21d exposure.

Identical specific activities of probe were added to each section (section 2.2.10.5). Note the even distribution of binding of probe throughout the hippocampus in A compared to the pattern of binding in B, which shows greater binding in CA1/2 and DG than in CA3/4.

CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus.

4.3.4 Both adrenalectomy and supraphysiological corticosterone replacement significantly increase glucocorticoid receptor mRNA in hippocampus of ST animals.

Both autoradiographs and dipped slides revealed obvious differences in total GR mRNA between the experimental groups (Figure 4.4, Figure 4.5). Adrenalectomy significantly increased GR mRNA by at least 30% in all hippocampal subfields while physiological corticosterone replacement restored levels to those of sham-operated controls (Figure 4.6).

In CA1 of the hippocampus, supraphysiological corticosterone replacement had no effect on GR mRNA levels. Interestingly, in CA2-CA4 and the dentate gyrus supraphysiological corticosterone replacement significantly increased GR mRNA compared to sham-operated controls (Figure 4.6).

In CA2-CA4 of hippocampus there are approximately 2-fold differences in GR mRNA between groups, whereas in CA1 or dentate gyrus the differences are approximately 30-40% (Figure 4.6).

Figure 4.4 (facing page): representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR gene to hippocampus of ST animals.

Binding of antisense probe appears greater in all hippocampal subfields in Adx and Adx/supra animals than in Adx/phys and Sham animals.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

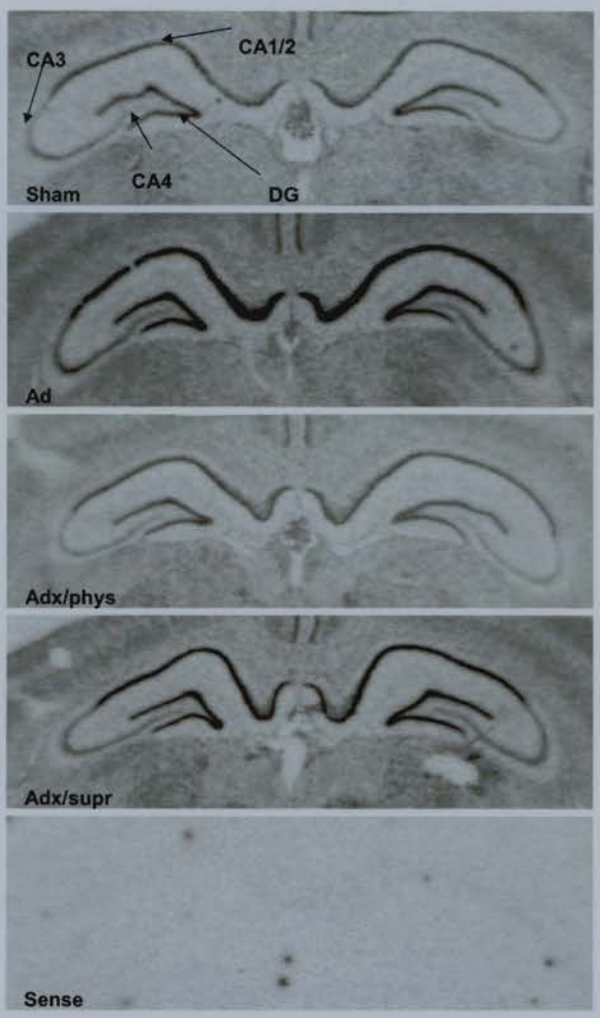
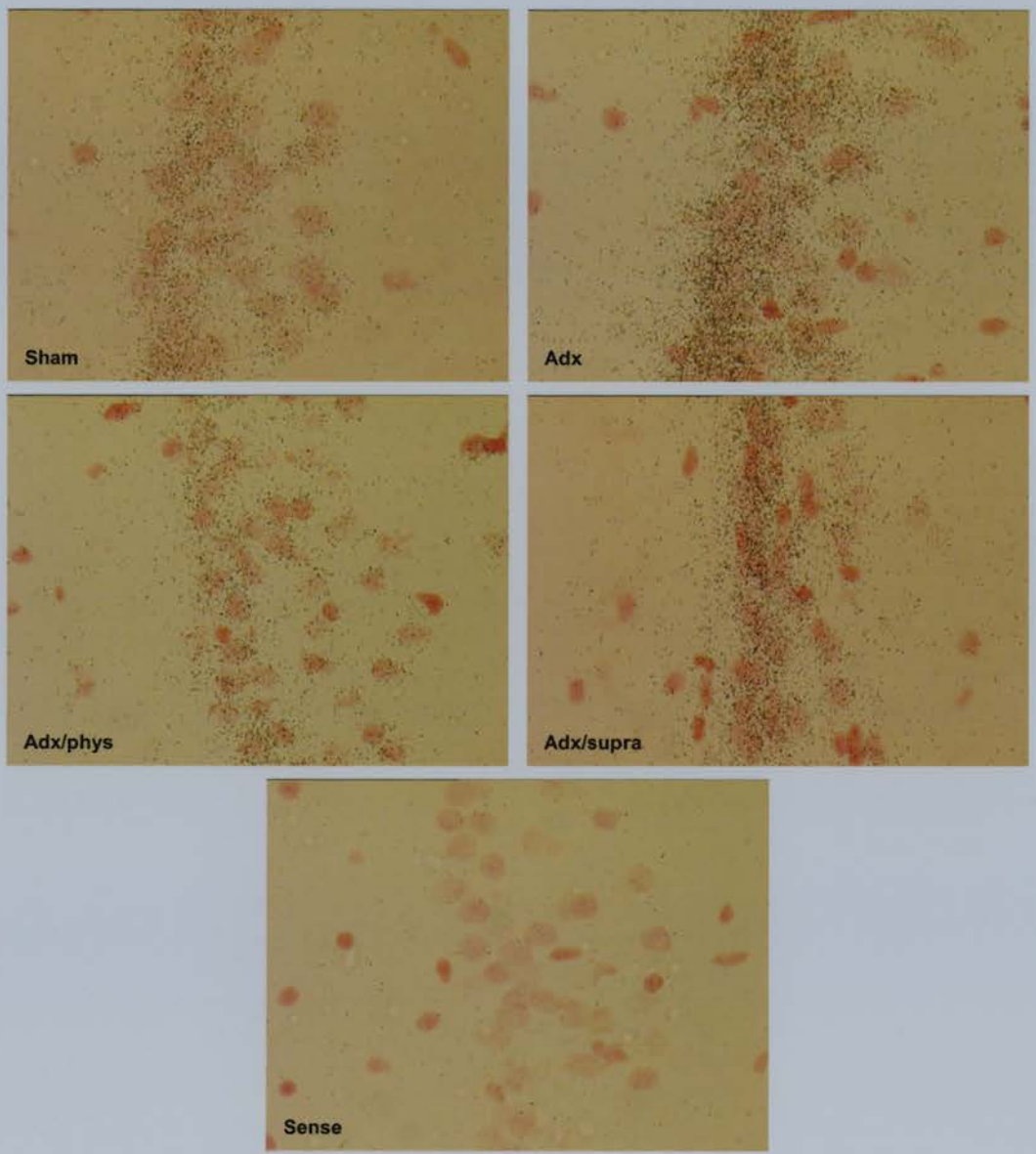

CA3
CA1/2
CA4
DG
Sham
Ad
Adx/phys
Adx/supr
Sense

Figure 4.5 (facing page): representative photomicrographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR gene to DG of ST animals.

More silver grains are present in Adx and Adx/supra animals than in Adx or Sham animals. Only a small amount of non-specific binding is seen with the corresponding sense control.

ST = short-term adrenalectomised, DG = dentate gyrus, Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.


Sham
Adx
Adx/phys
Adx/supra
Sense

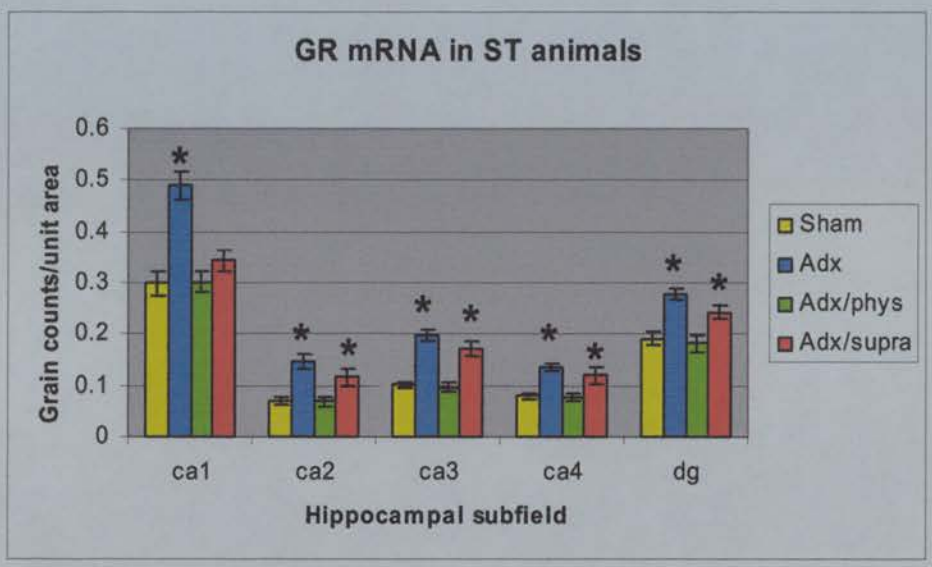


Figure 4.6: grain counting analysis of *in situ* mRNA hybridisation of a probe complementary for exons 5-9 of the GR gene to hippocampus of ST animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was a significant effect of treatment, with Adx animals (in all hippocampal subfields) and Adx/supra animals (in ca2-4 and dg) having significantly higher GR mRNA levels than sham-operated controls ($p<0.05$, indicated by *). Densitometric analysis of autoradiographs gave very similar results (data not shown). n=6 except sham n=7.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

4.3.5 Adrenalectomy increases glucocorticoid receptor mRNA expression in CA2 and CA4 of hippocampus in LT animals.

Quantitative analysis of silver grain distribution (Figure 4.7) showed a significant increase in GR mRNA in CA2 and CA4 of LT adrenalectomised animals compared to sham-operated controls (Figure 4.8 A). Analysis of autoradiographs (Figure 4.9) by densitometry (Figure 4.8 B) showed a very similar trend, although the differences did not reach statistical significance.

There appeared to be a trend in both the grain counting and densitometry data for adrenalectomy to increase GR mRNA levels in CA1 and CA3 (Figure 4.8). Conversely, there was no such trend in dentate gyrus (Figure 4.8). When analysed using Tukey's HSD Test the differences in CA1 and CA3 did not reach statistical significance. However, analysis using Fisher's LSD Test showed that in CA3 Adx animals had significantly higher GR mRNA than sham-operated controls (Figure 4.8).

Figure 4.7 (facing page): representative photomicrographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR gene to CA2 of LT animals.

More silver grains are present in sections from adrenalectomised animals.

LT = long-term adrenalectomised.CA2 = cornu ammonis region 2, Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

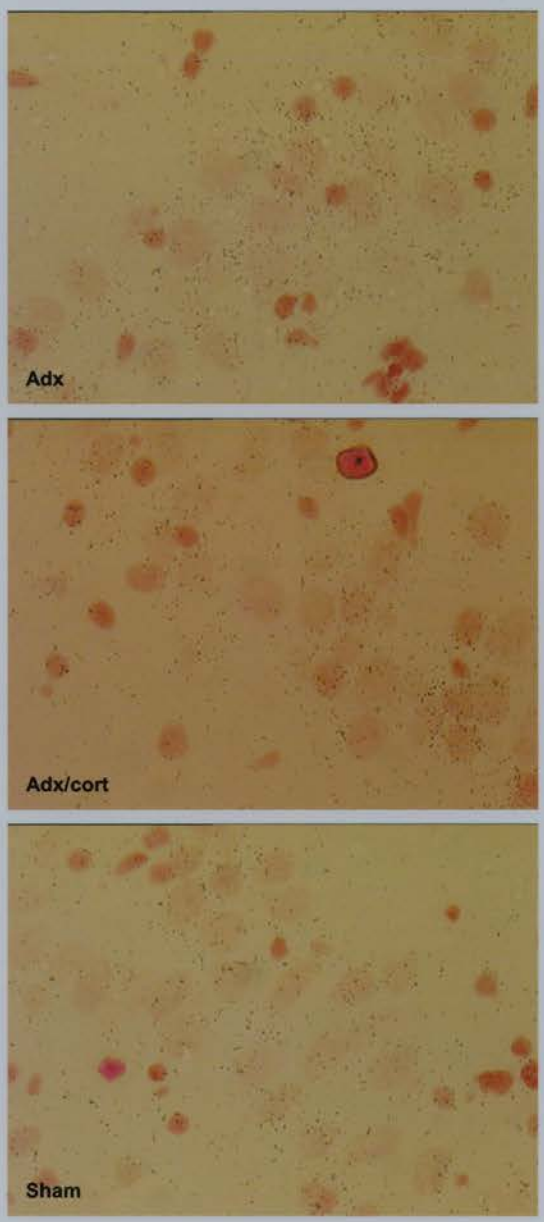
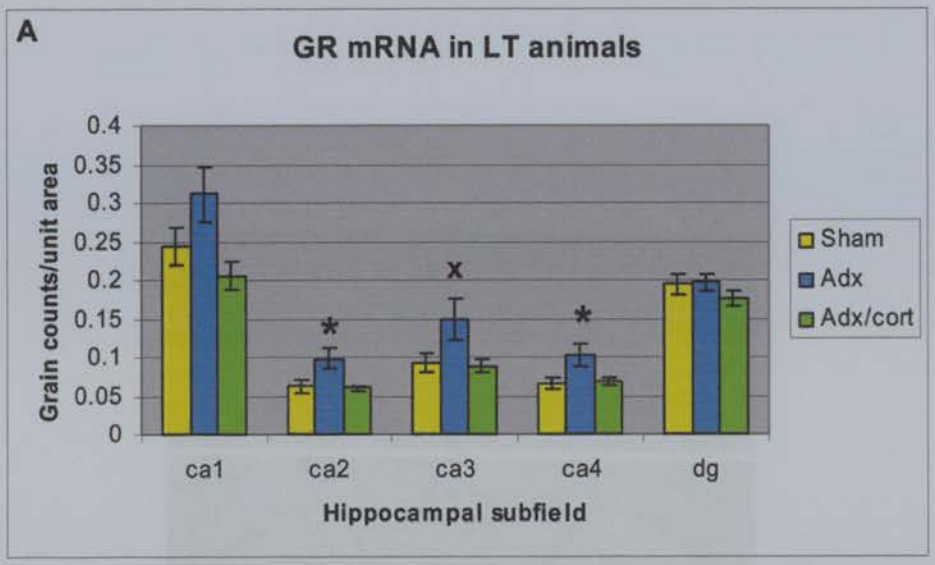

Adx
Adx/cort
Sham

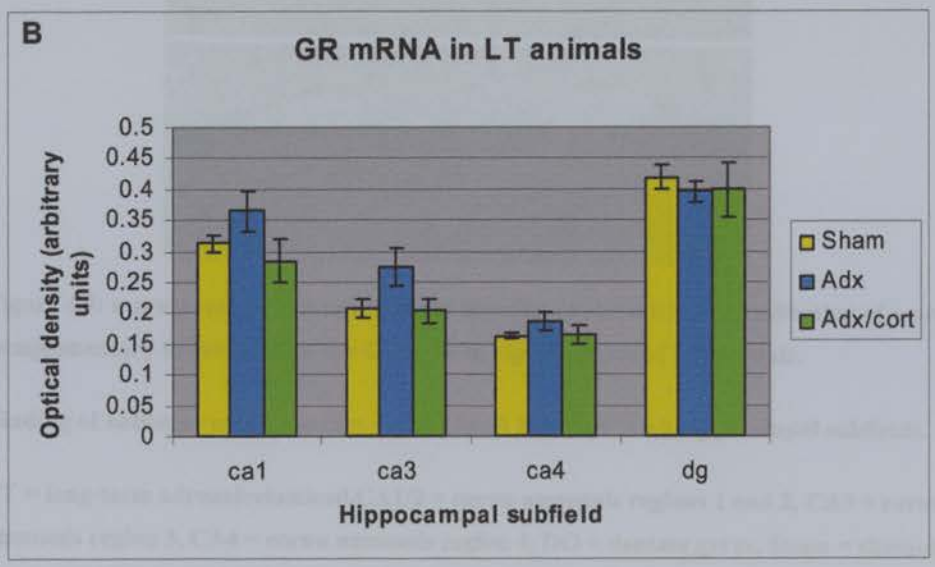
Figure 4.8 (facing page): analysis of *in situ* mRNA hybridisation of a probe specific for exons 5-9 of the GR gene to hippocampus of LT animals. Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

A: grain counting analysis. There was a significant effect of treatment, with Adx animals having significantly higher GR mRNA levels in ca2 and ca4 than sham-operated controls ($p<0.05$, indicated by *). Also, when analysed by Fisher's LSD Test Adx animals had significantly higher GR mRNA levels in ca3 than sham-operated controls ($p<0.05$, indicated by x).

B: densitometric analysis. There was no significant effect of treatment.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.


A
GR mRNA in LT animals
Grain counts/unit area
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
x
*
ca1
ca2
ca3
ca4
dg
Hippocampal subfield
Sham
Adx
Adx/cort


B
GR mRNA in LT animals
Optical density (arbitrary units)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ca1
ca3
ca4
dg
Hippocampal subfield
Sham
Adx
Adx/cort

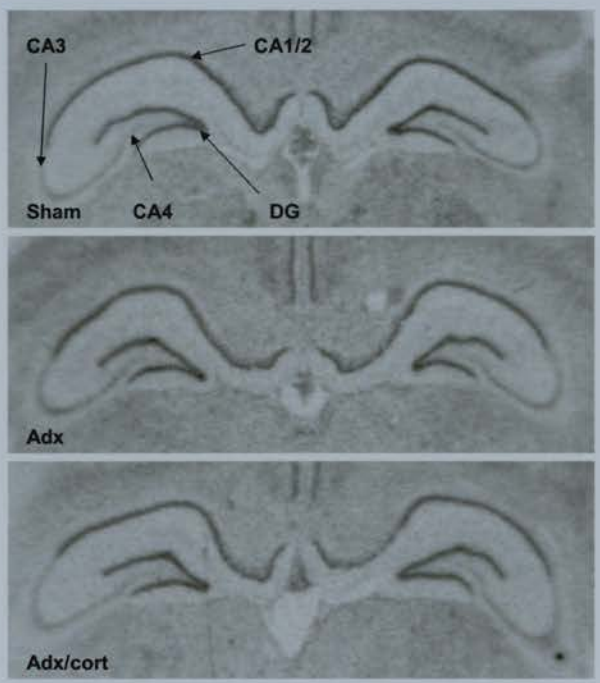


Figure 4.9: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR gene to hippocampus of LT animals.

Binding of antisense probe appears similar in all 3 groups in all hippocampal subfields.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

4.3.6 Exon 1_5 of the GR gene is expressed in all hippocampal subfields but its expression is not significantly altered by changes in glucocorticoid levels.

As shown previously (Chapter 3 and (McCormick et al., 2000) GR mRNA containing exon 1_5 is evenly distributed throughout the hippocampus (Figure 4.10, Figure 4.11).

In ST animals, although the means for the Adx and Adx/supra groups trend to be higher than the Sham and Adx/phys groups, mimicking the pattern of changes in total GR, expression of exon 1_5 was not significantly altered in any hippocampal subfield by glucocorticoid manipulation (Figure 4.12). Similarly, expression of exon 1_5 was not significantly altered by glucocorticoid manipulations in the LT animals (Figure 4.12).

Unfortunately, grain counting of the exon 1_5 experimental slides was not possible (section 2.2.10.8) and it was not possible to repeat the adrenalectomy experiments and carry out the necessary laboratory work in the time available.

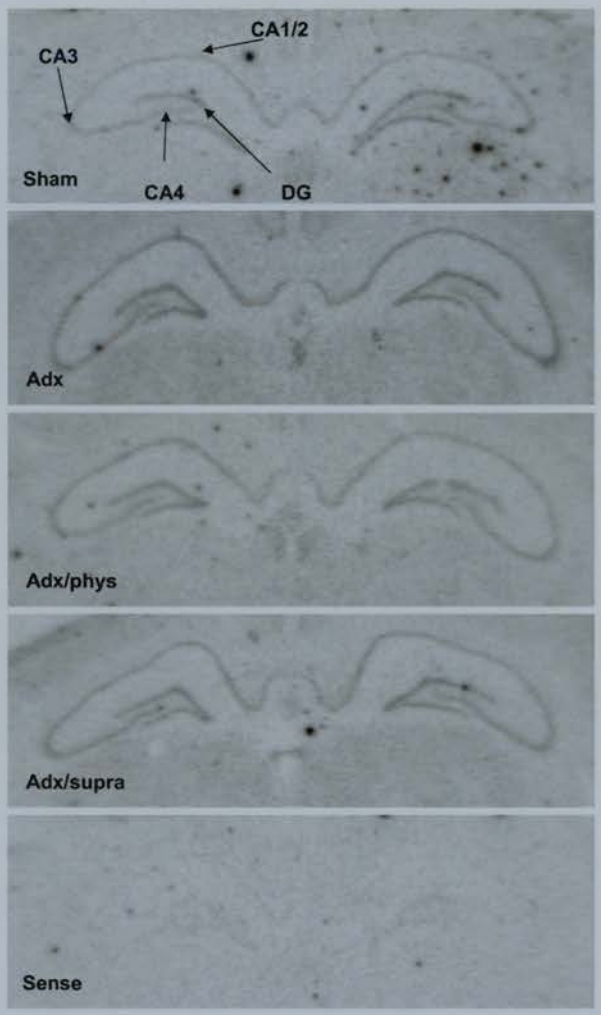


Figure 4.10: representative autoradiographs showing in situ mRNA hybridisation of a probe complementary to exon 1_5 of the GR gene to hippocampus of ST animals.

Similar binding of antisense probe is seen in all hippocampal subfields.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

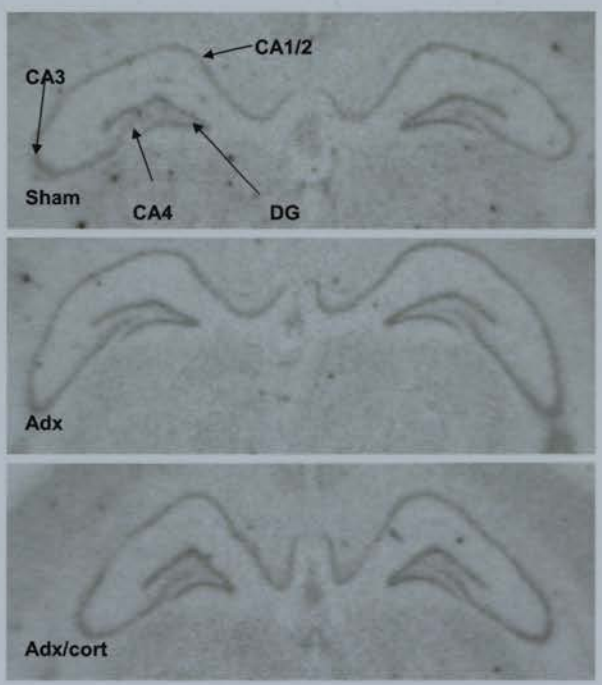


Figure 4.11: representative autoradiographs showing *in situ* mRNA hybridisation of a probe specific for exon 1_5 of the GR gene to hippocampus of LT animals.

Similar binding of antisense probe is seen in all hippocampal subfields. Representative sense control is shown in Figure 4.10.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

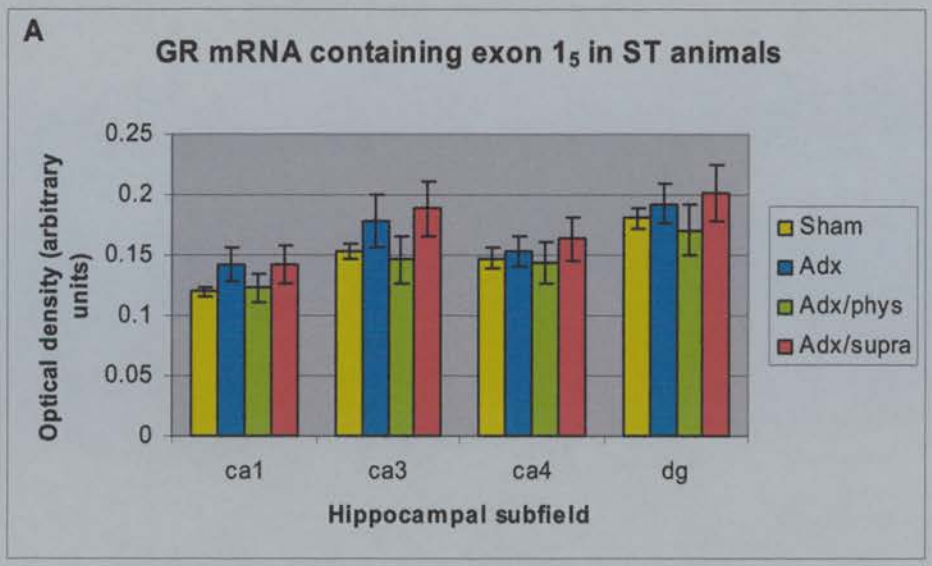
Figure 4.12 (facing page): densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_5 of the GR gene to rat hippocampus.

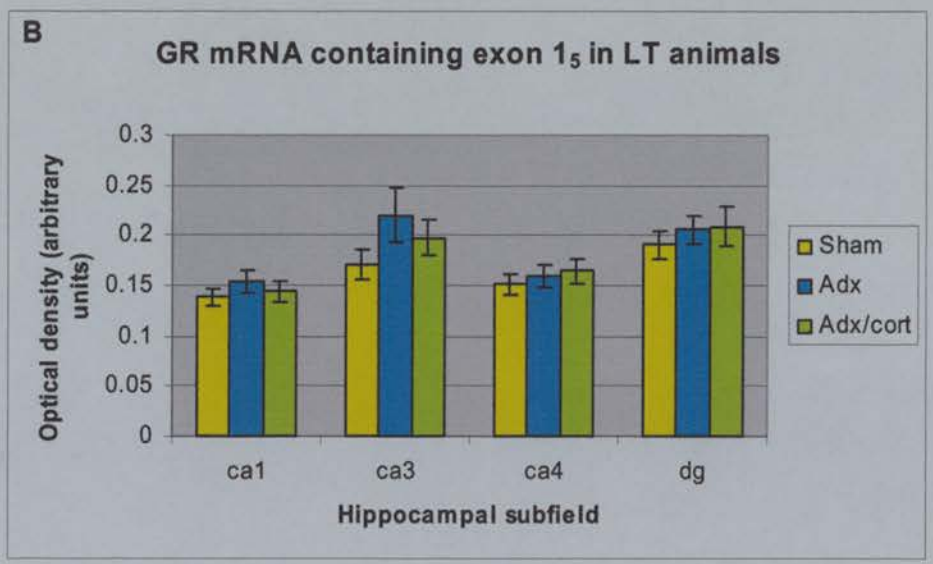
Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

A: ST animals. There was no significant effect of treatment. Adx/phys n=5, Adx/supra n=6, Adx n=6, Sham n=7.

B: LT animals. There was no significant effect of treatment. n=7 except sham n=8.

ST = short-term adrenalectomised. LT = long-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.


A
GR mRNA containing exon 1$_5$ in ST animals
Optical density (arbitrary units)
0.25
0.2
0.15
0.1
0.05
0
ca1
ca3
ca4
dg
Hippocampal subfield
Sham
Adx
Adx/phys
Adx/supra


B
GR mRNA containing exon 1$_5$ in LT animals
Optical density (arbitrary units)
0.3
0.25
0.2
0.15
0.1
0.05
0
ca1
ca3
ca4
dg
Hippocampal subfield
Sham
Adx
Adx/cort

4.3.7 Exon 1_7 of the GR gene is expressed in all hippocampal subfields but its expression is not significantly altered by changes in glucocorticoid levels.

GR mRNA containing exon 1_7 appears to be evenly distributed throughout the hippocampus (Figure 4.13, Figure 4.14).

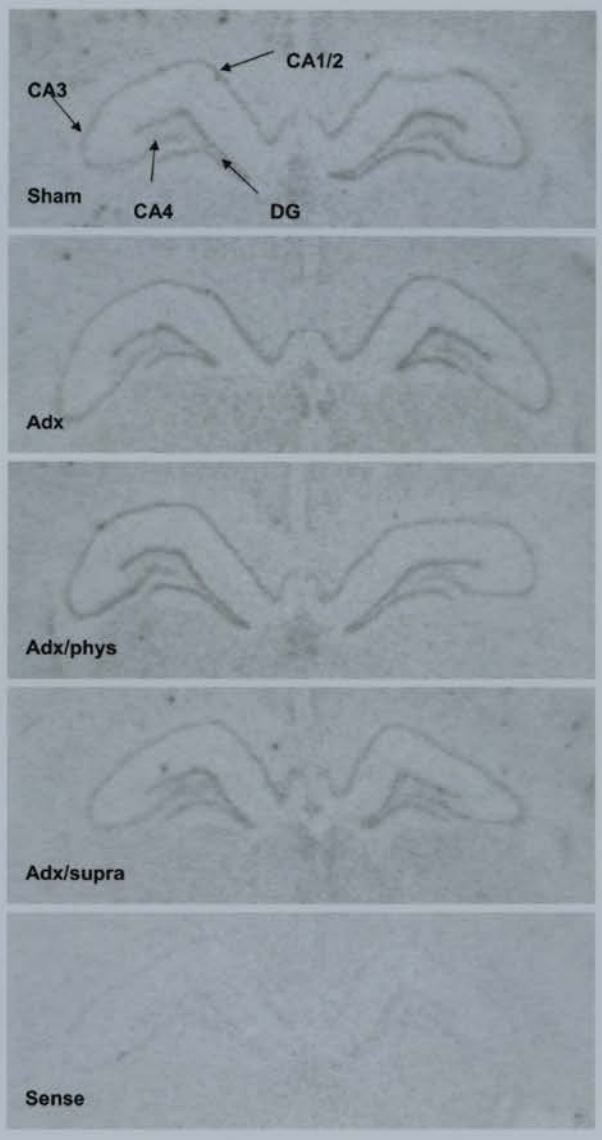
In both the ST and LT animals, the expression of GR mRNA containing exon 1_7 was not significantly altered by glucocorticoid manipulation (Figure 4.15, Figure 4.16) although in the ST animals there was a trend for the Adx and Adx/supra groups to have higher exon 1_7 expression than the Sham and Adx/phys groups in 3 out of the 4 hippocampal subfields, mimicking the pattern of changes in total GR mRNA (Figure 4.15). The densitometric analysis was repeated using a larger number of sample zones for each of these regions. This confirmed the previous results with no significant effect of treatment, although the observed trend persisted (data not shown).

Grain counting analysis gave similar results (data not shown).

Figure 4.13 (facing page): representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_7 of the GR gene to hippocampus of ST animals.

Similar binding of antisense probe is seen in all hippocampal subfields.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.


CA1/2
CA3
Sham
CA4
DG
Adx
Adx/phys
Adx/supra
Sense

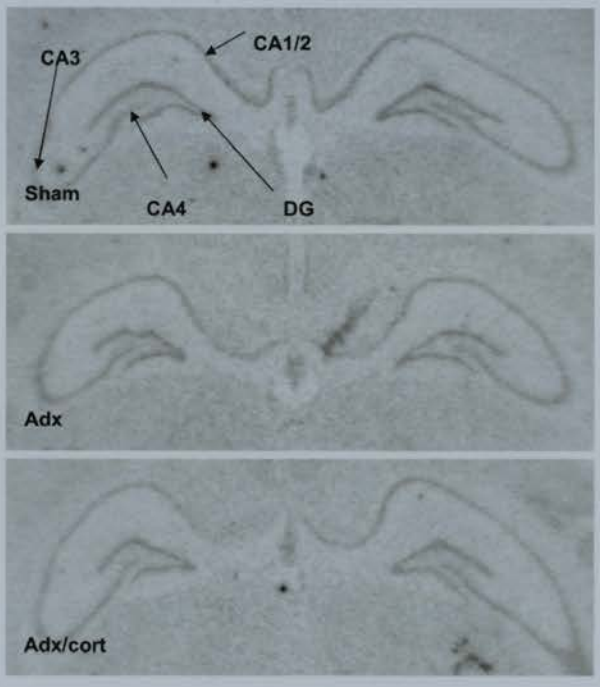


Figure 4.14: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_7 of the GR gene to hippocampus of LT animals.

Similar binding of antisense probe is seen in all hippocampal subfields in all experimental groups.

Representative sense control is shown in Figure 4.13

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

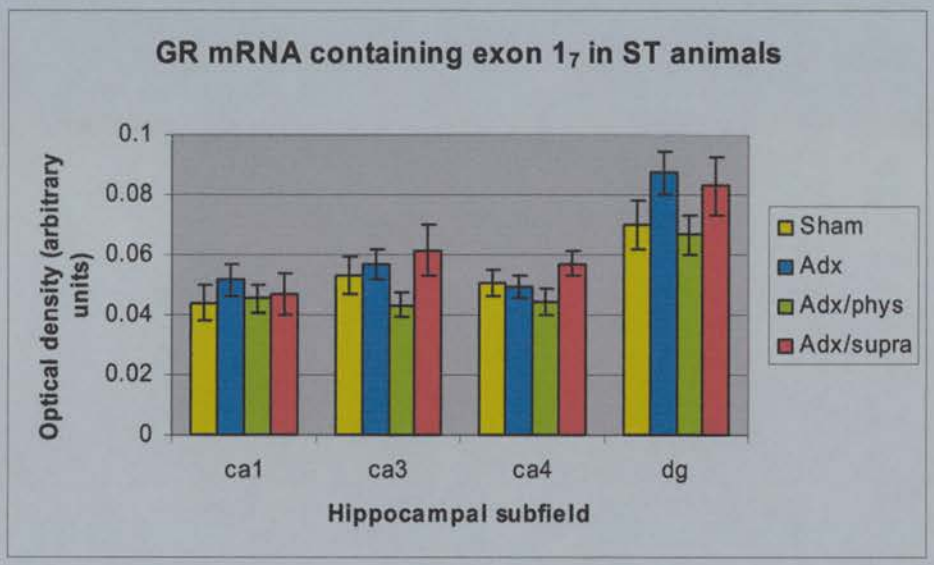


Figure 4.15: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_7 of the GR gene to hippocampus of ST animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was no significant effect of treatment. n=6 except Adx/supra n=5.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

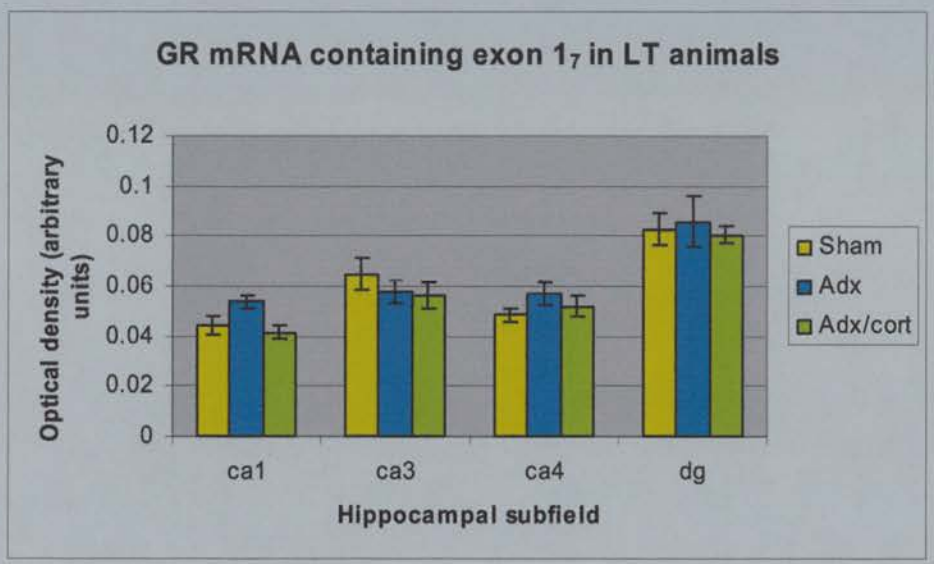


Figure 4.16: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_7 of the GR gene to hippocampus of LT animals.

There was no significant effect of treatment. n=8 except Adx n=7.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

4.3.8 Exon 1_{10} of the GR gene is expressed in all hippocampal subfields but its expression is not significantly altered by changes in glucocorticoid levels.

As described previously (Chapter 3 and (McCormick et al., 2000) GR mRNA transcripts containing exon 1_{10} are distributed similarly to the total population of GR mRNA transcripts in hippocampus (Figure 4.17F igure 4.18). Densitometric analysis indicated a trend in the ST animals for the Adx and Adx/supra groups to have higher levels of exon 1_{10} expression than the Sham and Adx/phys groups, mimicking the pattern of changes in total GR (Figure 4.19). However, these differences did not reach statistical significance (Figure 4.19) and there were no differences in exon 1_{10} expression in the LT animals (Figure 4.20).

Grain counting analysis gave similar results (data not shown).

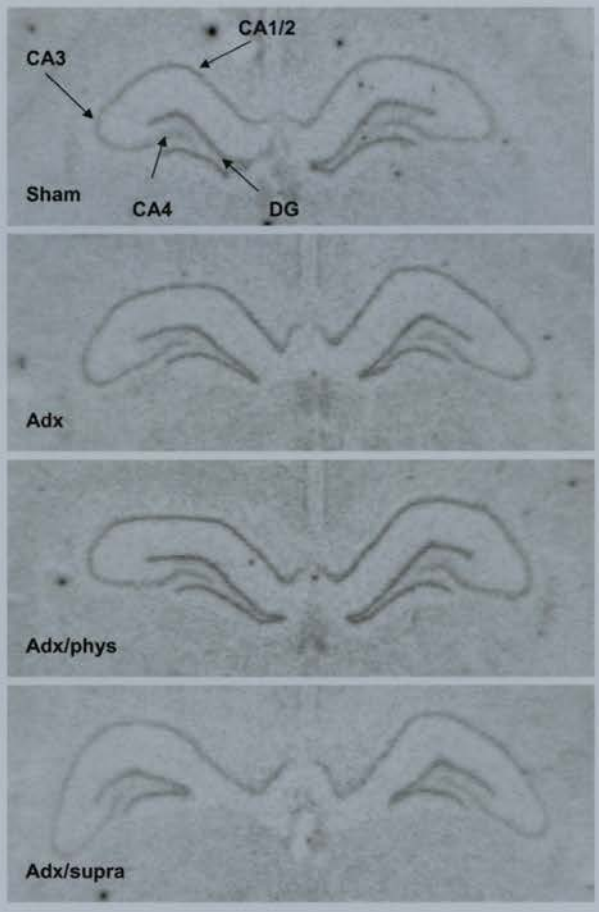


Figure 4.17: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to hippocampus of ST animals.

Greater binding of antisense probe is seen in CA1/2 and DG than in CA3 and CA4 in all experimental groups.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

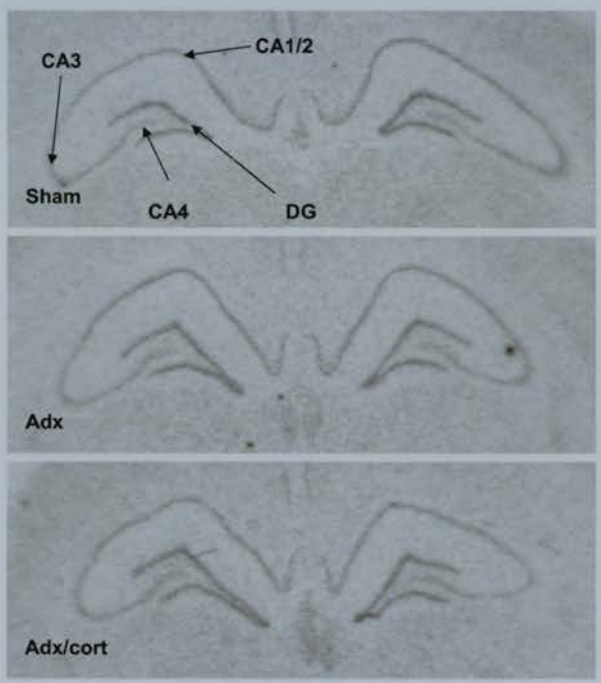


Figure 4.18: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to hippocampus of LT animals.

Greater binding of antisense probe is seen in CA1/2 and DG than in CA3 and CA4.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

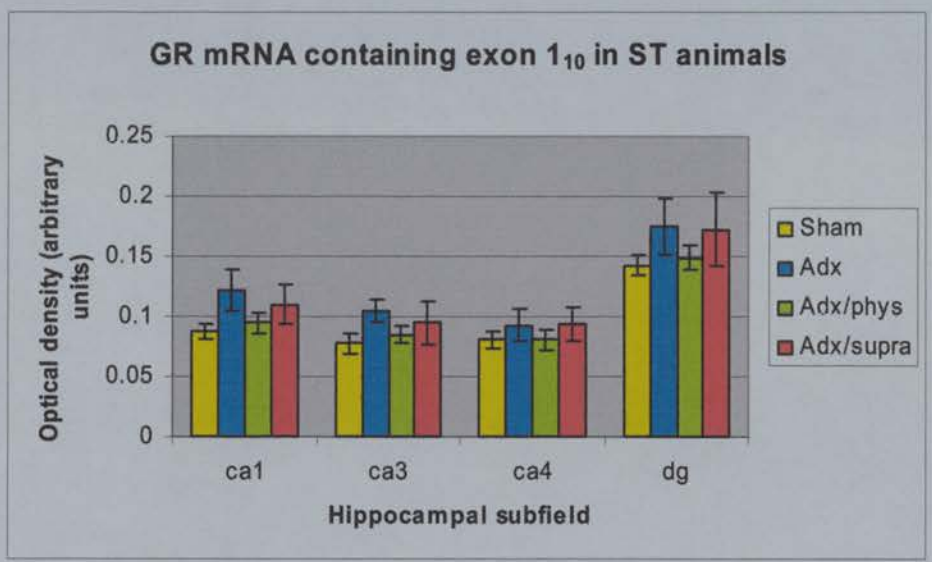


Figure 4.19: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to hippocampus of ST animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was no significant effect of treatment. n=6 except sham n=7.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

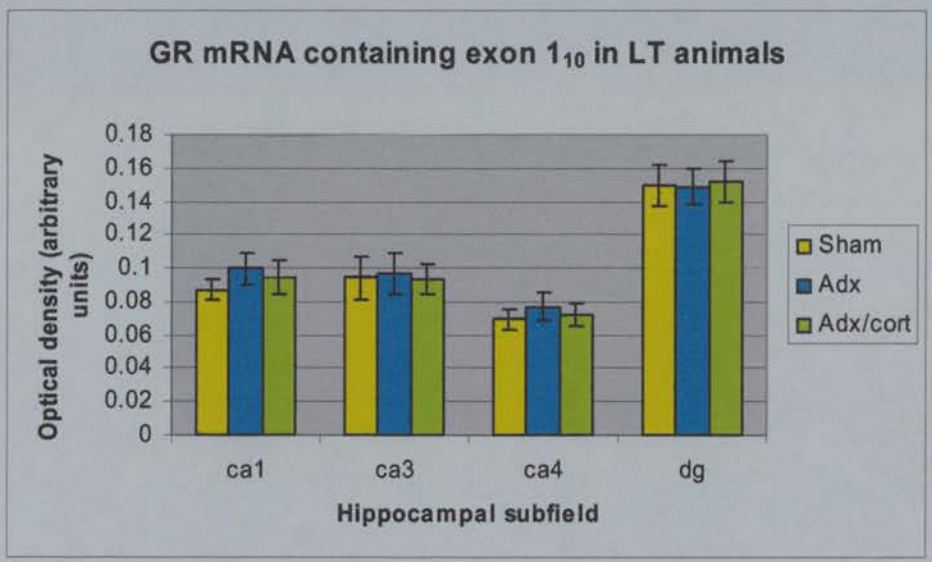


Figure 4.20: densitometric analysis of *in situ* mRNA hybridisation of a probe specific for exon 1_{10} of the GR gene in hippocampus of LT animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was no significant effect of treatment. n=8 except Adx n=7.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

4.3.9 Exon 1_{11} of the GR gene is expressed in all hippocampal subfields but its expression is not significantly altered by changes in glucocorticoid levels.

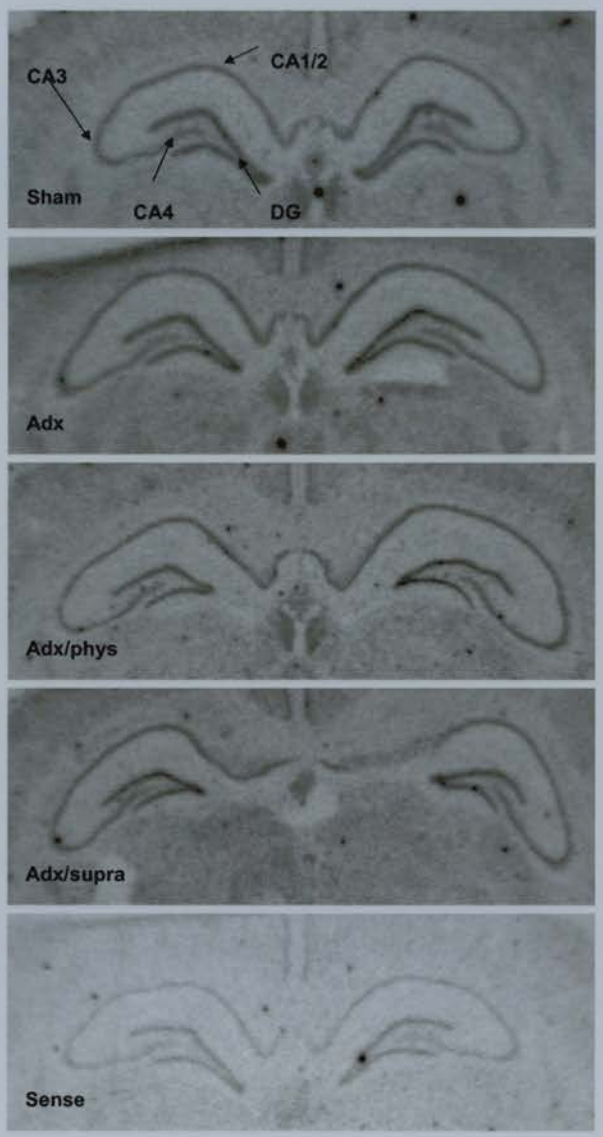
Examination of autoradiographs revealed that exon 1_{11} was evenly distributed in all hippocampal subfields (Figure 4.21F igure 4.22). Densitometric analysis of autoradiographs showed no significant effect of glucocorticoid manipulation on expression of GR mRNA transcripts containing exon 1_{11} in ST and LT animals (Figure 4.23, Figure 4.24). However, there was a trend for ST Adx and Adx/supra animals to have higher levels of exon 1_{11} expression in the hippocampus than Adx/phys animals or sham operated controls (Figure 4.23).

Grain counting analysis gave similar results (data not shown).

Figure 4.21 (facing page): representative autoradiographs showing *in situ* mRNA hybridisation with a probe specific for exon 1_{11} of the GR gene in hippocampus of ST animals.

Similar binding of antisense probe is seen in all hippocampal subfields.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.


CA3
CA1/2
Sham
CA4
DG
Adx
Adx/phys
Adx/supra
Sense

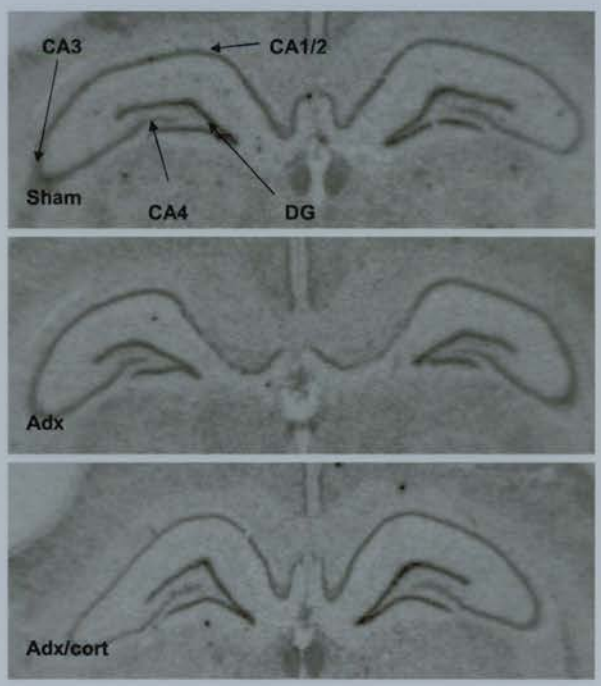


Figure 4.22: representative autoradiographs showing *in situ* mRNA hybridisation with a probe specific for exon 1_{11} of the GR gene in hippocampus of LT animals.

Similar binding of antisense probe is seen in all hippocampal subfields. Representative sense control is shown in Figure 4.21.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

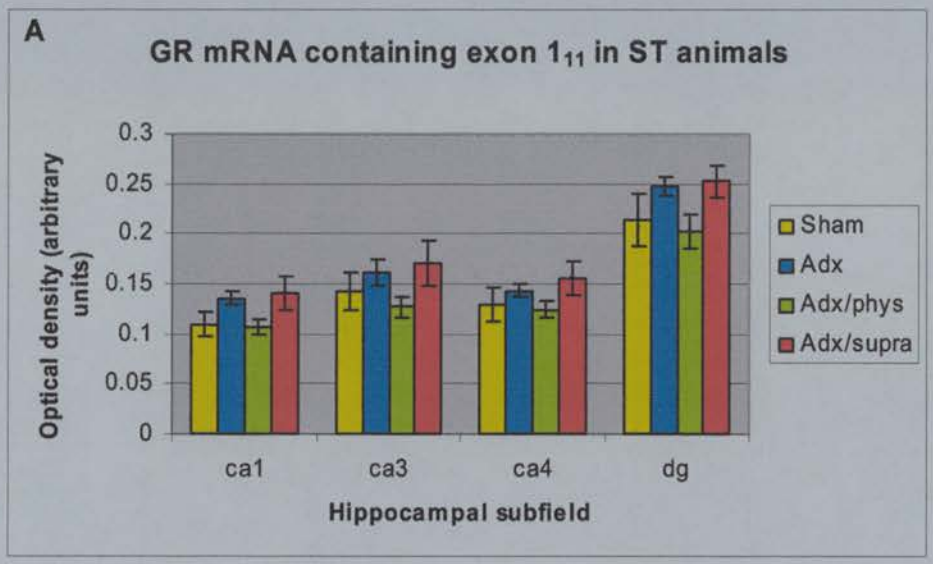


Figure 4.23: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{11} of the GR gene to hippocampus of ST animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was no significant effect of treatment. n=6 except Adx/supra n=4 and Adx n=5.

ST = short-term adrenalectomised. CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by daily injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by daily injection.

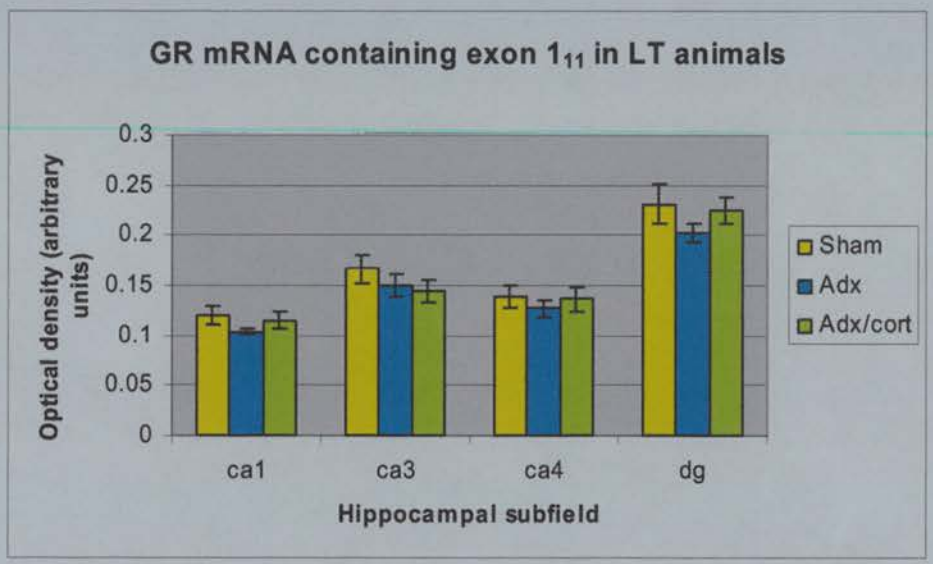


Figure 4.24: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{11} of the GR gene to hippocampus of LT animals.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was no significant effect of treatment. Adx n=6, Adx/cort n=7, Sham n=8.

LT = long-term adrenalectomised.CA1/2 = cornu ammonis regions 1 and 2, CA3 = cornu ammonis region 3, CA4 = cornu ammonis region 4, DG = dentate gyrus. Sham = sham-operated control, Adx = adrenalectomised, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet.

4.4 Discussion

It is well established that glucocorticoids are a major regulator of GR expression in the hippocampus (section 1.7.2.1) and that this regulation is likely to be at the level of transcription (Herman and Spencer, 1998; Rosewicz et al., 1988), although some post-translational regulation may also occur (Dong et al., 1988). Therefore, changes in the rate of GR gene transcription will be reflected in changes in mRNA levels. Here, I have examined whether hippocampal variant GR mRNA transcripts containing alternate exons 1 are differentially regulated by glucocorticoids. Although ST glucocorticoid manipulation significantly altered total GR mRNA levels (which were increased by adrenalectomy and supraphysiological glucocorticoid replacement and normalized by physiological glucocorticoid replacement), no significant changes were seen in GR mRNA containing any of the variant exons 1. However, for all the exons 1 a similar trend to the changes in total GR mRNA was noted. In contrast, LT glucocorticoid manipulation had a much smaller effect on total GR mRNA levels and also had no effect on levels of GR mRNA transcripts containing any of the variant exons 1.

The observed changes in total GR mRNA could not be accounted for by changes in expression of any of the variant GR mRNA transcripts studied here. There are three possible reasons why this might be so.

Firstly, the *in situ* mRNA hybridisation experiments might have failed to detect a genuine effect of changes in glucocorticoid levels on variant exon 1 expression. The riboprobes specific for the different exons 1 were relatively short, which means that the ratio of true signal to background "noise" due to non-specific binding of probe will be less than with a longer probe. This hypothesis is supported by the fact that although the densitometry data for all the individual exons 1 showed similar trends to the changes in total GR mRNA, these changes were not present in the grain counting data. Since densitometry examines a larger area of hippocampus than does grain

counting it assesses the overall changes in a large number of cells which will reduce the variability of the data.

Secondly, it is possible that this study was statistically underpowered. The number of animals in each group was chosen based on those used in previous experiments from our laboratory that had detected changes in total GR mRNA with adrenalectomy (Holmes et al., 1995b). Indeed, changes in total GR mRNA were demonstrated here. However, any glucocorticoid-induced changes in expression of the exons 1 studied here must be much smaller than those in total GR mRNA and thus would require a larger number of animals in each group to detect. Another, more quantitative technique such as RNase protection analysis might have been more appropriate than *in situ* hybridisation for this study. However, such techniques do not offer any anatomical detail. Ideally, one would like to repeat this series of experiments with a larger number of animals in each group and perhaps with redesigned probes (e.g. with higher specific activity) to overcome some of the problems discussed above. Another possible approach would be to split the hippocampus in half longitudinally when harvesting the tissue and use half of it for *in situ* mRNA hybridisation and half of it for total RNA extraction and RNase protection analysis.

Thirdly, the increase in GR mRNA levels seen with adrenalectomy may be due to an increase in expression of transcripts containing another variant exon 1 not studied here (known or novel) or to small changes in expression of all the individual exons 1 that are not significant when analysed in isolation. Either of these possibilities is intriguing, since the hippocampus specific exon 1_7 is specifically upregulated by neonatal handling. However, the results presented here suggest that none of the exons 1 studied is dramatically regulated by glucocorticoids. It is possible that changes in expression of transcripts containing exon 1_6 might account for the changes as they make up 17-20.7% of the hippocampal GR mRNA population (McCormick et al., 2000). Unfortunately it is not possible to generate a probe for *in situ* mRNA hybridisation to detect this exon (section 3.2.2), so it could not be included in this study. Changes in the expression of exon 1_6 with glucocorticoid manipulation could be investigated by RNase protection analysis and this would be an interesting line of future enquiry.

It is interesting to compare the above results with those from studies on other nuclear receptors. The rat mineralocorticoid (Kwak et al., 1993; Zennaro et al., 1995) and human (Flouriot et al., 1998) and mouse (Kos et al., 2000) oestrogen receptors express multiple variant mRNAs with alternate 5' untranslated exons 1. Variant human (Flouriot et al., 1998; Osterlund et al., 2000) and mouse (Kos et al., 2000) oestrogen receptor mRNAs are expressed in a tissue and region-specific manner. In the case of the mineralocorticoid receptor, variant exons 1 are associated with the activity of alternate promoters (Zennaro et al., 1995; Zennaro et al., 1996) and show tissue and region-specific differences in their relative expression (Kwak et al., 1993; Vazquez et al., 1998). Furthermore, the α and β variants of the rat mineralocorticoid receptor are specifically upregulated in the hippocampus by adrenalectomy (Vazquez et al., 1998). If the hypothesis that none of the glucocorticoid receptor exons 1 is individually regulated by glucocorticoids is correct, then there is an interesting difference in the regulation of these members of an otherwise similar gene family.

A significant increase in GR mRNA levels was seen in all hippocampal subfields with ST adrenalectomy, while the effects of LT adrenalectomy were of lesser magnitude and were confined to CA2 and CA4. In previously published studies, adrenalectomy of up to 8d duration caused significant increases in hippocampal GR mRNA (Herman et al., 1989a; Herman and Spencer, 1998; Holmes et al., 1995b; Reul et al., 1989; Sheppard et al., 1990) and GR binding sites (Lowy, 1989; Spencer et al., 1991), while after adrenalectomy of greater than 14d duration hippocampal GR mRNA levels returned to normal (Holmes et al., 1995b; Reul et al., 1989). Although increased hippocampal total GR mRNA (Kalinyak et al., 1987) and GR binding sites (Reul et al., 1987a) have been reported 2 weeks after adrenalectomy, the balance of evidence suggests an initial rise in hippocampal GR after adrenalectomy with a gradual fall back towards resting levels over a period of approximately 2 weeks, in agreement with the data presented here.

In the experiments described here physiological corticosterone replacement in adrenalectomised animals restored hippocampal total GR mRNA levels to those of control animals, in agreement with previous studies showing that corticosterone

replacement orally over 5d (Sheppard et al., 1990) or by subcutaneous pellets over 4 (O'Donnell et al., 1995), 6 (Spencer et al., 1991), or 7d (Reul et al., 1987a) either abolishes (O'Donnell et al., 1995; Sheppard et al., 1990; Spencer et al., 1991) or significantly reduces (Reul et al., 1987a) the adrenalectomy-induced rise in hippocampal GR and reduces the number of GR binding sites in adrenally intact animals (Sapolsky et al., 1984b; Sapolsky and McEwen, 1985; Spencer et al., 1991). The data presented here are also in agreement with the results of studies using dexamethasone administration, which abolishes the adrenalectomy-induced rise in GR mRNA (Herman et al., 1989a; Holmes et al., 1995b; Peiffer et al., 1991a; Reul et al., 1989) and variably reduces hippocampal GR mRNA in adrenally intact animals (Sheppard et al., 1990).

In contrast, ST supraphysiological corticosterone replacement increased total GR mRNA levels in CA3/4 and DG, suggesting the effects are region specific and dose-dependent. A dose-dependent effect of both dexamethasone (Reul et al., 1987a) and corticosterone (O'Donnell et al., 1995; Spencer et al., 1991) on hippocampal GR has been previously reported. However, studies using corticosterone replacement at "supraphysiological" doses, either orally (Tornello et al., 1982), by injection (Patacchioli et al., 1998; Peiffer et al., 1991a; Sapolsky et al., 1984b; Sapolsky and McEwen, 1985) or by subcutaneous pellet (O'Donnell et al., 1995; Sheppard et al., 1990; Spencer et al., 1991; Tornello et al., 1982) report unaffected (Sheppard et al., 1990) or reduced hippocampal GR mRNA (Peiffer et al., 1991a) and reduced hippocampal GR binding sites (Patacchioli et al., 1998; Sapolsky et al., 1984b; Sapolsky and McEwen, 1985; Tornello et al., 1982). None of these studies reported increased hippocampal total GR mRNA with supraphysiological corticosterone replacement.

In fact, in this experiment radioimmunoassay showed that the ST animals receiving supraphysiological corticosterone replacement do not have significantly higher plasma corticosterone levels than those receiving physiological replacement. This is surprising given the tenfold difference in the doses given and the different effects on hippocampal GR in the two groups. However, the plasma for the assay was obtained approximately 17h after the last injection of corticosterone so the levels obtained are

likely to be close to the lowest levels present in the animals over a 24h period, which might account for their similarity. An indication of the likely maximum levels of corticosterone achieved in the plasma of these animals and their physiological effect could be obtained by assessing the effect of the replacement regimens on the bodyweight of the animals, since glucocorticoid excess is known to cause catabolism and negative nitrogen balance in animals with Cushing's disease (Bush, 1991) and 4 weeks of chronic stress has been shown to cause weight loss in rats (Mizoguchi et al., 2001). Animals in the supraphysiological replacement group tended to lose more weight than the physiologically-replaced group, although the difference did not reach statistical significance. However, as the experimental period was only 3d it is possible that insufficient time had elapsed for the two groups to lose significantly different amounts of weight and thus it is hard to draw firm conclusions from these results.

Increased hippocampal total GR mRNA with supraphysiological corticosterone replacement is an interesting and novel finding. This may not have been detected in previous studies due to differences in methodology. The replacement regimen used here mimics the natural diurnal rhythm of corticosterone release in the rat (Spencer et al., 1993), which is abolished in studies using corticosterone pellets. Also, none of the previous studies used a similar dose of corticosterone to that used here and many used different routes of corticosterone administration. The oral bioavailability of corticosterone is unknown and the bioavailability of corticosterone given by different parenteral routes e.g. intraperitoneal (Peiffer et al., 1991a) or subcutaneous (Sapolsky et al., 1984b) may vary. Furthermore, different strains of rat may show different HPA responses to glucocorticoids (Oitzl et al., 1995) and none of the previous studies used the Wistar strain, so the response of this strain may be unique. Further research is required to establish if other rat strains respond in a similar way to the same corticosterone replacement regimen and to elucidate any physiological effects of the increased hippocampal GR e.g. on cognitive function or behaviour (section 1.9.2.6). It is tempting to speculate that at very high levels of circulating corticosterone the GR in hippocampus become saturated, requiring an increased number of receptors to transduce a greater glucocorticoid signal and increase negative feedback from the

HPA axis. Under these circumstances, an increased number of activated receptors would be present in the nucleus and thus would be more likely to decrease production of factors involved in HPA axis control such as CRH and POMC by associating with negative GREs (section 1.11.2). Also, if a subset of hippocampal neurones were to upregulate GR in response to elevated glucocorticoid levels while the majority downregulate them, the neurones with elevated GR levels might undergo apoptosis while those with low levels survive. Indeed, glucocorticoid upregulation of GR has been associated with T cell apoptosis (Tonko et al., 2001) and it is possible that the reduction in GR levels seen during thymocyte maturation (Ranelletti et al., 1987) could be due to the deletion of a subset of thymocytes that highly express GR. This hypothesis is supported by the observation that different thymocyte subpopulations have different levels of GR (Wiegers et al., 2001). Neither the rate of apoptosis nor changes in GR levels in specific subpopulations of cells were addressed in the studies of hippocampal GR regulation described here. Further experiments to quantify these variables would be extremely interesting.

Interestingly, a transient increase in hippocampal GR has been reported in rats undergoing 7 hours of restraint stress (Fujikawa et al., 2000), suggesting that increases in circulating corticosterone might upregulate hippocampal GR in these animals. However, chronic stress causes downregulation of hippocampal GR over 7d (Paskitti et al., 2000), 14d (Kitraki et al., 1999), 18d (Kim et al., 1999), 3 week (Sapolsky et al., 1984b) and 4 week (Mizoguchi et al., 2001) experimental periods. Furthermore, stress clearly affects other brain regions than the hippocampus, e.g. the medial prefrontal cortex (Diorio et al., 1993) and in the hippocampus stress modulates the expression of a wide range of receptor proteins, e.g. growth hormone receptor (Fujikawa et al., 2000), the gamma-amino butyric acid A receptor (Cullinan and Wolfe, 2000), MR (Fujikawa et al., 2000) and the serotonin 2C (Holmes et al., 1995a) and 7 (Yau et al., 2001c) receptors. Since serotonin regulates GR in hippocampal neurons *in vivo* (Seckl et al., 1990; Yau et al., 1997b) and *in vitro* (Erdeljan et al., 2001; Hery et al., 2000; Mitchell et al., 1990b; Mitchell et al., 1992) and other neurotransmitters regulate hippocampal GR (Tritos et al., 1999), any effects of stress on hippocampal GR levels are a combination of glucocorticoid-

mediated effects and those acting via other signalling cascades. Consequently, results of experiments investigating stress-induced changes in hippocampal GR are not directly comparable with those described here.

The results described in this chapter suggest that although there is specific "static" regulation of GR mRNAs containing at least one variant exon 1 (exon 1_7, (McCormick et al., 2000)) by neonatal programming, there is no specific dynamic regulation of individual hippocampal GR mRNA variants by glucocorticoids in the adult animal. This finding led to the question of whether the same was true in the liver, where neonatal programming of GR levels has been shown to affect the relative abundance of variant GR mRNAs (McCormick et al., 2000). The results of experiments investigating this are described in Chapter 5.

5 The effect of glucocorticoid manipulations on the expression of the GR gene and alternate exons 1 in the liver

5.1 Introduction

The repression of GR expression by glucocorticoids in the hippocampus has been discussed (Chapter 4). Also, evidence suggests that glucocorticoids regulate GR expression in the liver: GR levels are increased by adrenalectomy and reduced by glucocorticoid replacement in mice (Svec, 1988; Svec et al., 1989) and hepatic GR is downregulated by stress (Alexandrova and Farkas, 1992). Furthermore, previous work in our laboratory has shown that hepatic GR levels may be permanently "programmed" by prenatal exposure to dexamethasone in a region-specific manner (Nyirenda et al., 1998). Some evidence suggests that "programming" of hepatic GR levels may be mediated by changes in variant exon 1 usage (McCormick et al., 2000).

The experiments in this chapter were designed to investigate whether glucocorticoid regulation of hepatic GR involves differential regulation of variant exon 1 expression in adult rats. The exons 1 studied were 1_6, 1_{10} and 1_{11} since GR mRNA transcripts containing these exons form the majority of the GR mRNA in the liver (McCormick et al., 2000). In addition, exon 1_5 was also included in the study since semiquantitative RT-PCR had suggested it may be present at levels comparable to exon 1_{11} in rat liver (section 3.3.1).

5.2 Experimental Design

Tissues used in the experiments described in this chapter were obtained from the same animals described in Chapter 4.

Two complementary techniques were used to study variant exon 1 expression:

(a) Solution hybridisation (section 2.2.11). This technique is highly quantitative and allows determination of both the total amount of GR mRNA present in the tissue and the proportion of the total which contains any individual exon 1.

(b) *In situ* mRNA hybridisation (section 2.2.10). This technique gives anatomical resolution and permits separate but semiquantitative analysis of expression of individual exons 1 in periportal and perivenous regions of the hepatic acinus.

5.2.1 Design of RNase protection analysis

The RNase protection assay has been previously described and has been used to investigate tissue-specificity of alternate exon 1 usage (McCormick et al., 2000). Briefly, the principle of the technique is that a GR mRNA transcript containing a particular exon 1 and the total population of GR mRNAs may be simultaneously quantified using a riboprobe complementary to both the exon 1 of interest and part of exon 2 of the GR gene (Figure 5.1). To determine whether GR mRNA levels were regulated by glucocorticoids, an actin cRNA probe was included as an internal control (section 2.2.11.2).

In all cases, quantitation of RNase protection analyses was performed by phosphoimager (section 2.2.11.3).

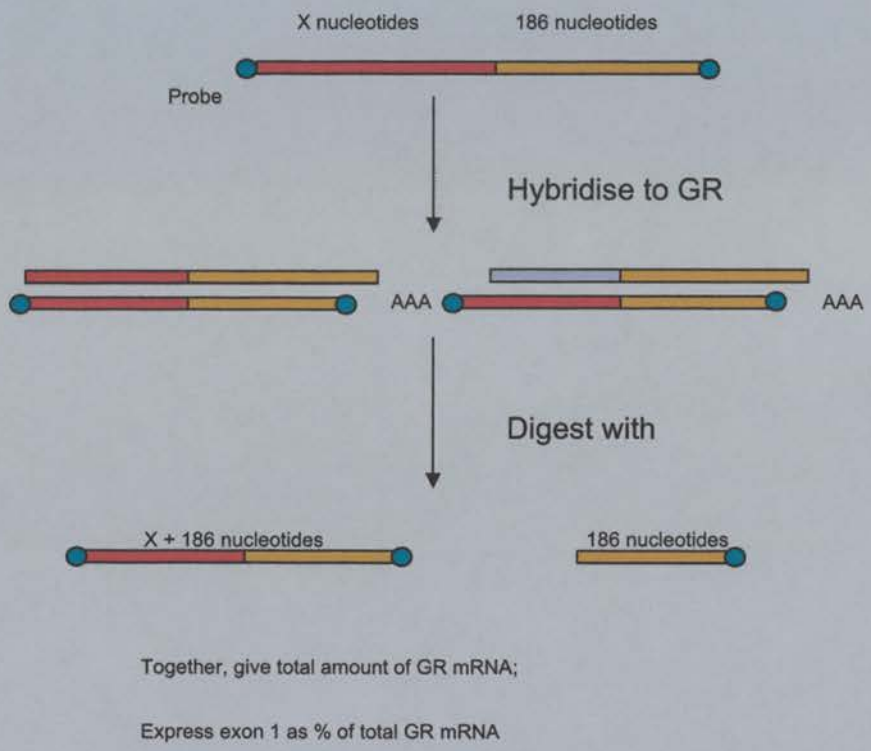


Figure 5.1: principle of the RNase protection assay.

A radiolabelled cRNA probe is incubated with total RNA from the tissue of interest. A common portion of 186 nucleotides is complementary to the 5' end of exon 2 of GR and can hybridise with all GR mRNAs. The 3' region of the probe can hybridise to the exon 1 of interest if GR mRNA transcripts containing it are present in the sample, producing a longer protected fragment. Hybridised regions of the probe are protected from RNase digestion and can be resolved on a denaturing polyacrylamide gel. The sum of the intensities of the bands produced by these two fragments gives the total amount of GR mRNA in the sample. The amount of the exon 1 of interest present can be expressed as a percentage of total GR mRNAs. Adapted from McCormick J. (2000), PhD thesis, University of Edinburgh.

5.3 Results

5.3.1 Optimisation of RNase protection analysis.

Quantitation of RNA using RNase protection analysis requires

(i) the cRNA to be in excess of the target mRNA

(ii) total digestion of the unbound probe.

To establish the linear range of the assay and titrate the amount of RNase necessary to completely digest excess cRNA, a pilot experiment was performed using different amounts of total RNA and a range of concentrations of the RNase provided in the HybSpeed RPA Kit II (AMS Biotechnology). In addition, to establish whether dissolution in formamide affected the assay, a comparison was made between RNA dissolved in water and formamide. Varying quantities of liver RNA were hybridised to probes complementary to exon 1_{10}-exon 2 of GR mRNA (exon 1_{10} probe) and actin mRNA (as an internal control).

Figure 5.2 shows a representative autoradiograph: autoradiographs of RNase protection analysis gels are used for illustrative purposes throughout this chapter and Chapter 6 since they provide good resolution and clear definition of bands. However, analysis of the assays was performed using a phosphoimager (section 2.2.11.3) as this allows rapid and accurate quantification. An example phosphoimager image, corresponding to the autoradiograph shown in Figure 5.2, is shown in Figure 5.3.

Phosphoimager analysis of the band intensities for the exon 1_{10} protected fragment in both the formamide and water suspended RNA (lanes 10 to 17, Figure 5.2) confirmed that the assay was approximately linear with respect to RNA added between 12.5 and 50μg in both cases (Figure 5.4).

In addition to the expected fragments, several fragments were obtained with sizes between that of the exon 2 and exons 1+2 protected fragments (186 nucleotides and 306 nucleotides respectively for exon 1_{10}). In particular, one band appeared of a

similar size to the full-length actin probe (Figure 5.2). Similar fragments had been detected previously in other experiments in this laboratory (McCormick J, PhD thesis, University of Edinburgh). As the same bands appeared in RNase protection analyses using a "sense" exon 1_{10} transcript (McCormick J, PhD thesis, University of Edinburgh), they were attributed to artefacts due to the high GC content in the probes causing them to form secondary structures.

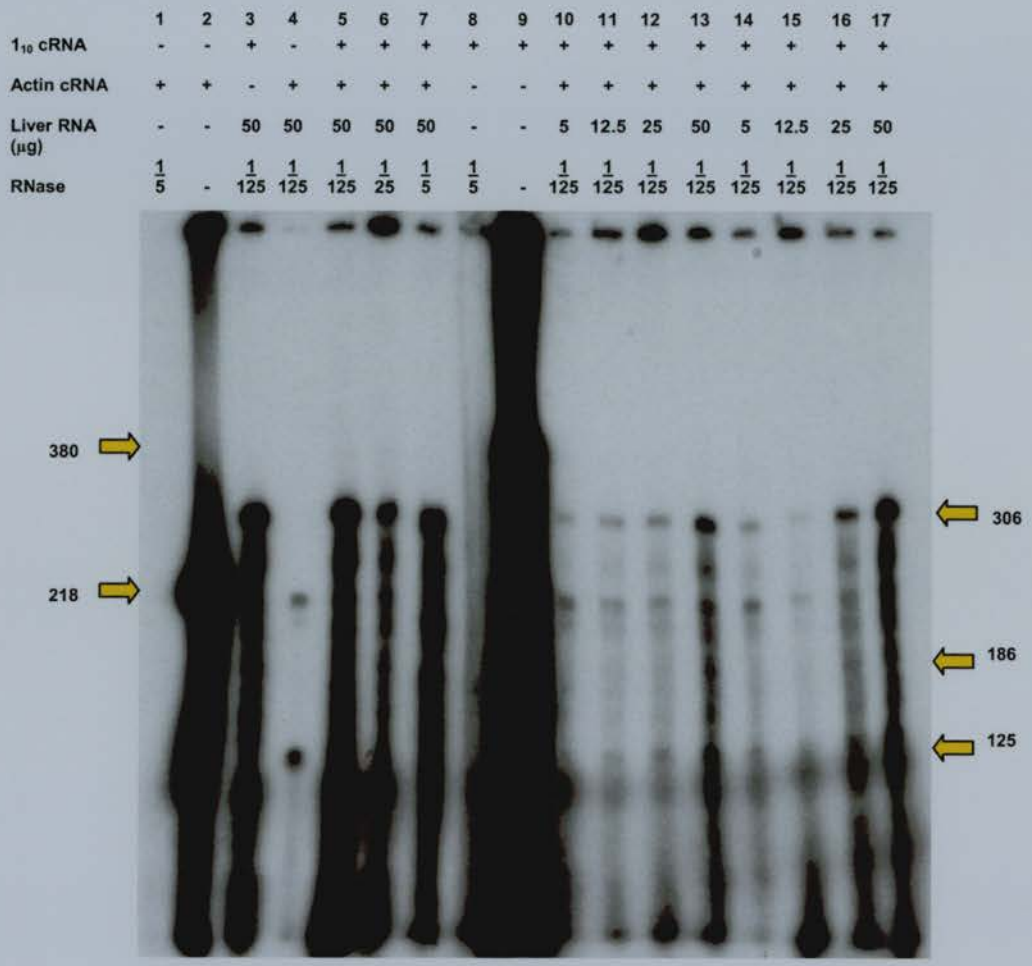
Using the RNase provided in the kit, complete digestion of the unhybridised full-length exon 1 and actin probes was achieved by using a 1/5 dilution of RNase (lanes 1 and 8, Figure 5.2). RNase-free control probes remained undigested (lanes 2 and 9, Figure 5.2). When hybridised to liver RNA suspended in formamide, the full-length exon 1_{10} and actin probes were completely digested by RNase at 1/5 dilution (lane 7, Figure 5.2). However, use of lower dilutions of RNase resulted in incomplete digestion of the full-length actin probe (lanes 4-6 and 10-17, Figure 5.2) although the full-length exon 1_{10} probe was completely digested with RNase at 1/125 dilution (lanes 3, 5 and 10-17, Figure 5.2).

To see if RNase T1 from different manufacturers was comparable, another RNase protection analysis was performed using RNase T1 provided by Roche Diagnostics along with the AMS RNase. In this analysis, use of a 1/5 dilution of Roche RNase degraded the protected fragments in addition to excess cRNA while a 1/25 dilution did not (lanes 1-3, Figure 5.5).

In view of these results, subsequent RNase protection analyses were performed using 50μg of liver RNA (suspended in formamide) per reaction and a 1/25 dilution of Roche Diagnostics RNase.

Figure 5.2 (facing page): autoradiograph showing pilot RNase protection analysis of GR mRNA transcripts containing exon 1_{10} of the GR gene in rat liver total RNA.

The cRNA probe, amount of liver RNA and RNase concentration in each reaction are indicated at the top of the figure. The cRNA probes were complementary to exon 1_{10}/exon 2 of the GR gene (1_{10} cRNA) or actin (actin cRNA). The reactions in lanes 3-7 and 10-13 used RNA suspended in deionised formamide, the remainder of the lanes used RNA suspended in RNase-free water. Arrows indicate the 380 nucleotide full-length 1_{10} cRNA, the 218 nucleotide full-length actin cRNA, the 306 nucleotide protected fragment produced by GR mRNA containing both exon 1_{10} and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_{10} and the 125 nucleotide protected fragment produced by actin mRNA. Exposure is 7d.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1₁₀ cRNA - - + - + + + + + + + + + + + + +
Actin cRNA + + - + + + + - - + + + + + + + +
Liver RNA (µg) - - 50 50 50 50 50 - - 5 12.5 25 50 5 12.5 25 50
RNase 1/5 - 1/125 1/125 1/125 1/25 1/5 1/5 - 1/125 1/125 1/125 1/125 1/125 1/125 1/125 1/125
380
218
306
186
125

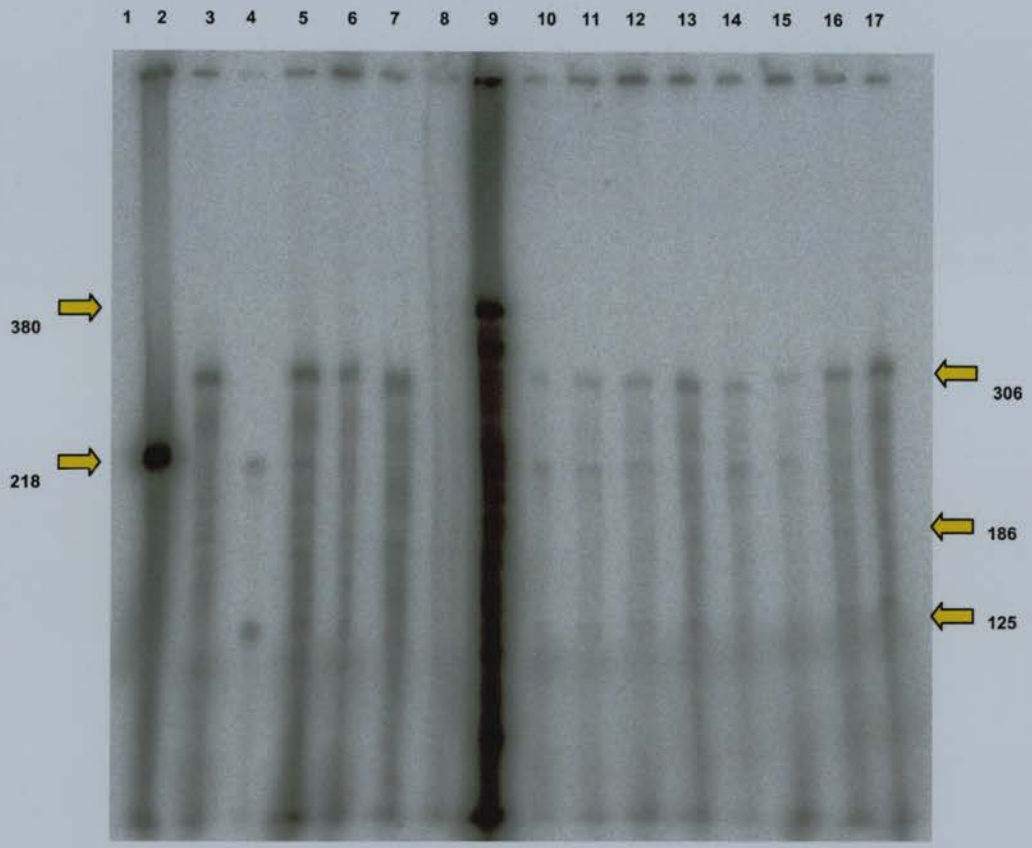


Figure 5.3: example phosphoimager image used for quantitation of RNase protection analysis.

Image corresponds to the autoradiograph shown in Figure 5.2 and is an 8h exposure. For contents of lanes 1-17 see Figure 5.2.

Arrows indicate the 380 nucleotide full-length 1_{10} cRNA, the 218 nucleotide full-length actin cRNA, the 306 nucleotide protected fragment produced by GR mRNA containing both exon 1_{10} and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_{10} and the 125 nucleotide protected fragment produced by actin mRNA.

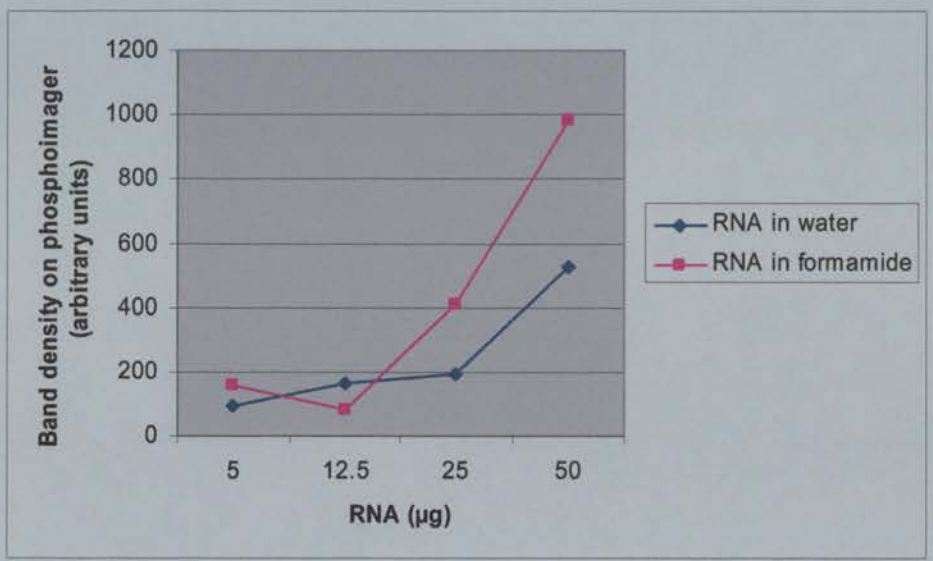


Figure 5.4: quantitation of RNase protection assay shown in Figure 5.2.

Density of bands representing the exon 1_{10} protected fragment was measured using a phosphoimager (section 2.2.11.3) and plotted against the amount of liver RNA added to the reaction. The results of the assay were approximately linear for both RNA suspended in water and RNA suspended in formamide between 5 and 50µg RNA.

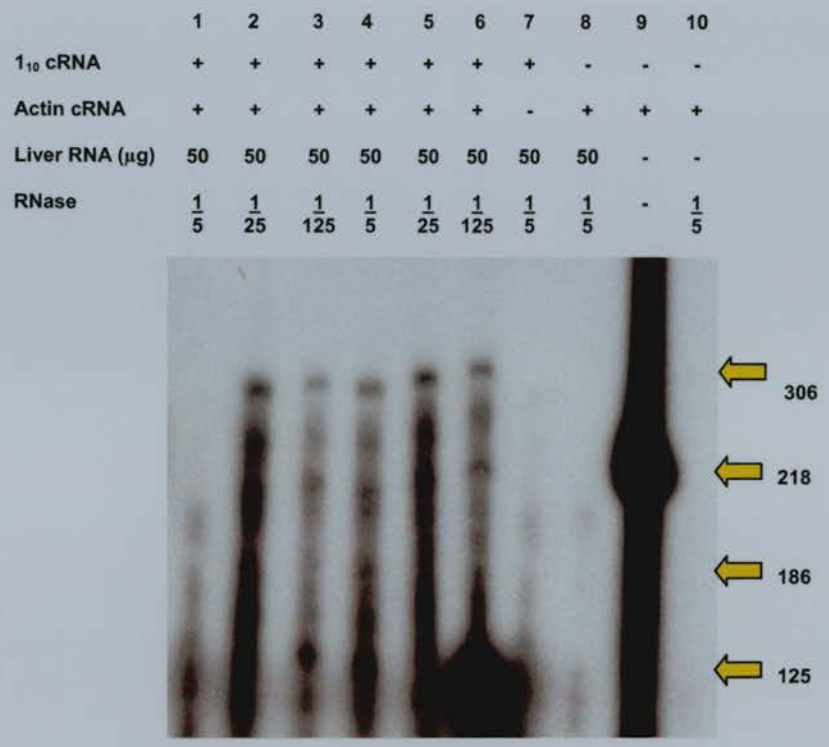


Figure 5.5: autoradiograph showing second pilot RNase protection analysis of GR mRNA transcripts containing exon 1_{10} of the GR gene in rat liver total RNA.

The cRNA probe, amount of liver RNA and RNase concentration in each reaction are indicated at the top of the figure. The reactions in lanes 1-3, 7, 8 and 10 contained RNase T1 provided by Roche Diagnostics, while lanes 4-6 contained RNase T1 provided by AMS Biotechnology. The cRNA probes were complementary to exon 1_{10}/exon 2 of the GR gene (1_{10} cRNA) or actin (actin cRNA). All RNA was dissolved in deionised formamide. Arrows indicate the 218 nucleotide full-length actin cRNA, the 306 nucleotide protected fragment produced by GR mRNA containing both exon 1_{10} and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_{10} and the 125 nucleotide protected fragment produced by actin mRNA. Exposure is 7d.

5.3.2 Effects of glucocorticoid manipulation on expression of GR mRNA containing exons 1_5, 1_6, 1_{10} and 1_{11} in the livers of ST animals

RNase protection analysis showed that there was a significant (approximately 50%) decrease in the absolute amount of GR mRNA containing exon 1_{10} in the Adx/phys group compared to the Adx animals, although the proportion of GR mRNA containing exon 1_{10} (approximately 60-70%) was unaffected by changes in glucocorticoid levels (Figure 5.6, Figure 5.7).

RNase protection analysis showed that approximately 14% of GR mRNA in the liver contained exon 1_6 (Figure 5.8, Figure 5.9). Glucocorticoid manipulation had no effect on either the proportion of the total GR mRNA population containing exon 1_6 or the absolute amount of GR mRNA containing exon 1_6 in the liver (Figure 5.9). However, there were strong trends for a significant effect of treatment on both these values ($p=0.07$ and $p=0.09$ respectively) with glucocorticoid replacement tending to lower exon 1_6 expression (Figure 5.9).

Expression of GR mRNA containing exons 1_5 and 1_{11} was below the level of detection in total liver RNA from any experimental group (Figure 5.10, Figure 5.11), indicating that they must make up less than 1-2% of the total GR mRNA population (McCormick et al., 2000).

Comparison of the Adx and Adx/phys groups had shown an approximately 2x decrease in exon 1_{10} expression and a strong trend for the same effect on exon 1_6 expression. Together, these exons 1 are present in approximately 75% of the total GR mRNA. To determine the magnitude of the change in total GR mRNA with glucocorticoid manipulation in the livers of ST animals, data from the individual RNase protection analyses was combined. This analysis showed no difference in levels of total GR mRNA between adrenalectomised and sham-operated control animals (Figure 5.12). The difference between the adrenalectomy with replacement groups and the adrenalectomised group did not reach statistical significance, although both replacement groups showed significantly (approximately 50% and

40% respectively) lower levels of GR mRNA than the sham-operated controls (Figure 5.12).

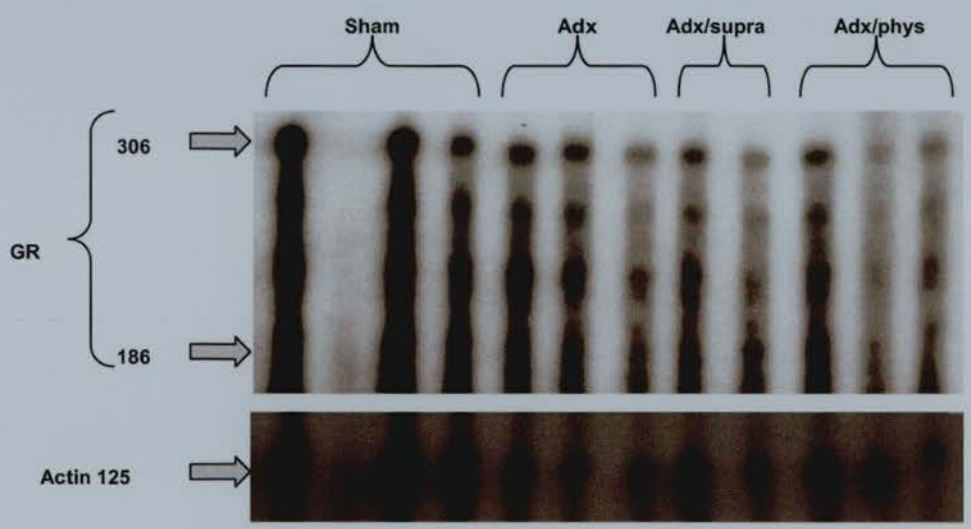


Figure 5.6: representative autoradiograph showing RNase protection analysis of expression of exon 1_{10} of the GR gene in liver of ST animals.

Each lane shows the product of an RPA reaction carried out on 50μg of liver RNA from a single animal. Arrows indicate the 306 nucleotide protected fragment produced by GR mRNA containing both exon 1_{10} and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_{10} and the 125 nucleotide protected fragment produced by actin mRNA. Upper panel shows a 7d autoradiograph exposure, lower panel is an overnight exposure.

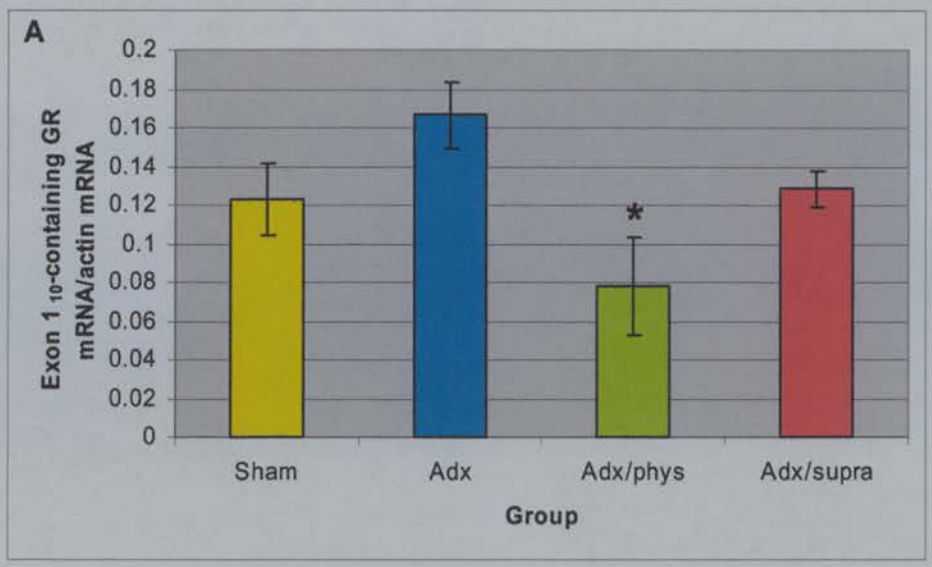
Figure 5.7 (facing page): RNase protection analysis of GR mRNA containing exon 1_{10} in livers of ST animals.

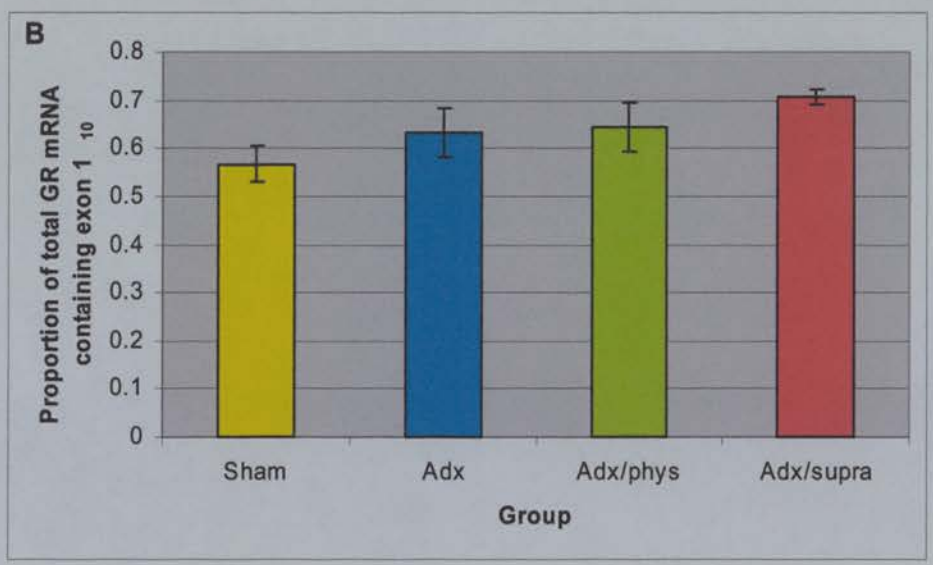
Data are presented as mean ± SEM.

A: total amount of GR mRNA containing exon 1_{10}, expressed as a ratio to actin mRNA.D ata were analysed with ANOVA and Tukey's HSD test. There was a significant effect of glucocorticoid manipulation, with a reduction in exon 1_{10} expression in the Adx/phys group compared to the Adx group ($p<0.05$, indicated by *).

B: proportion of GR mRNA containing exon 1_{10}. ANOVA showed no significant effect of treatment.

Adx/phys n=5, Adx/supra n=4, Adx n=5, Sham n=6.


A
Exon 1 10-containing GR mRNA/actin mRNA
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
Sham
Adx
Adx/phys
Adx/supra
Group


B
Proportion of total GR mRNA containing exon 1 10
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Sham
Adx
Adx/phys
Adx/supra
Group

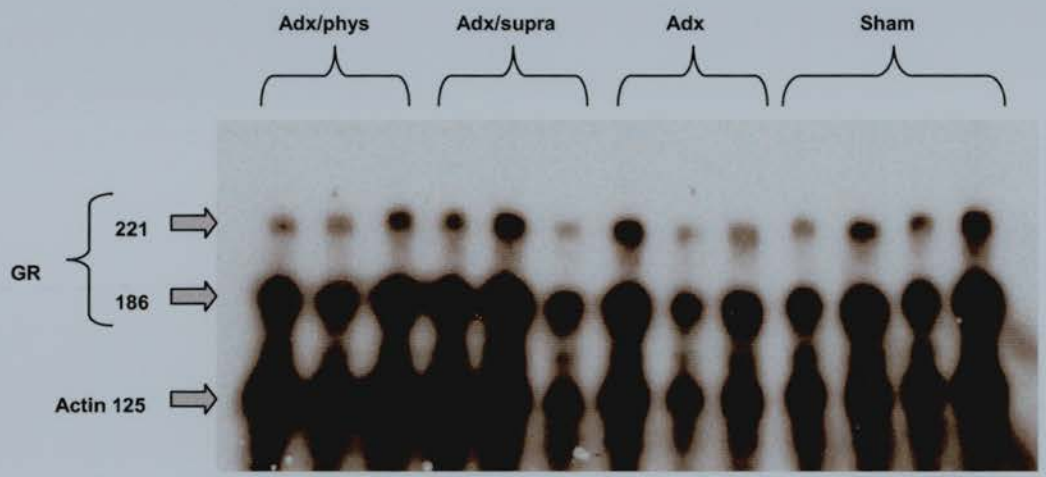
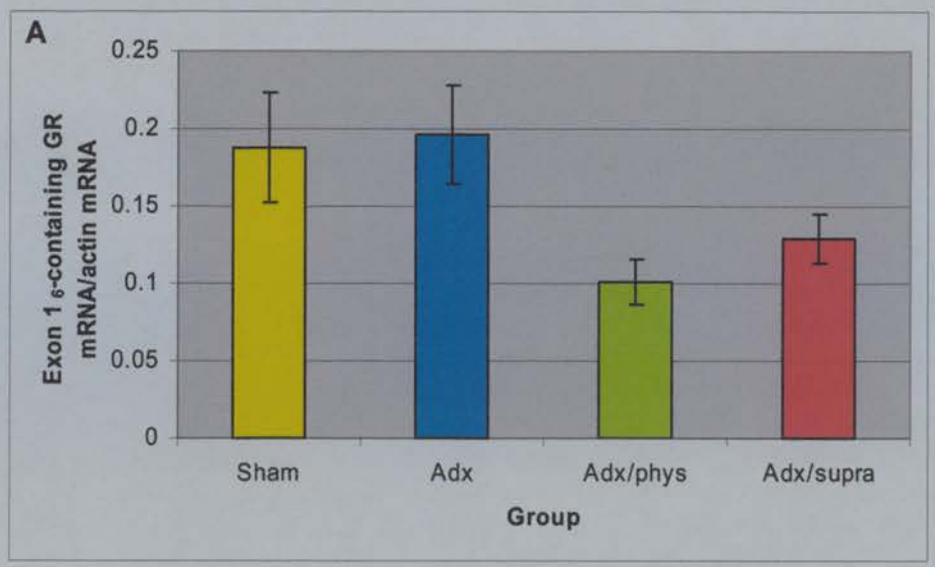


Figure 5.8: representative autoradiograph showing RNase protection analysis of expression of exon 1_6 of the GR gene in liver of ST animals.

Each lane shows the product of an RPA reaction carried out on 50μg of liver RNA from a single animal. Arrows indicate the 221 nucleotide protected fragment produced by GR mRNA containing both exon 1_6 and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_6 and the 125bp protected fragment produced by actin mRNA.

Autoradiograph shown is a 5d exposure.

Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement.



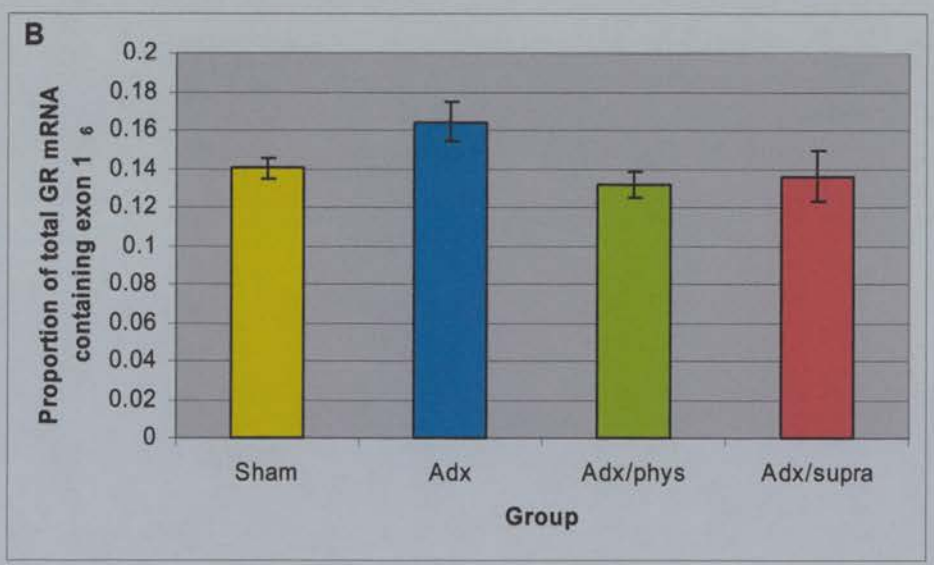


Figure 5.9: RNase protection analysis of GR mRNA containing exon 1_6 in livers of ST animals.

Data are presented as mean ± SEM.

A: Total amount of GR mRNA containing exon 1_6 expressed as a ratio to actin mRNA. ANOVA showed no significant effect of treatment group on exon 1_6 expression. n=5 except Adx/phys n=6 and Sham n=8.

B: Proportion of total GR mRNA containing exon 1_6. ANOVA showed no significant effect of treatment group on exon 1_6 expression. n=6 per group except sham where n=7.

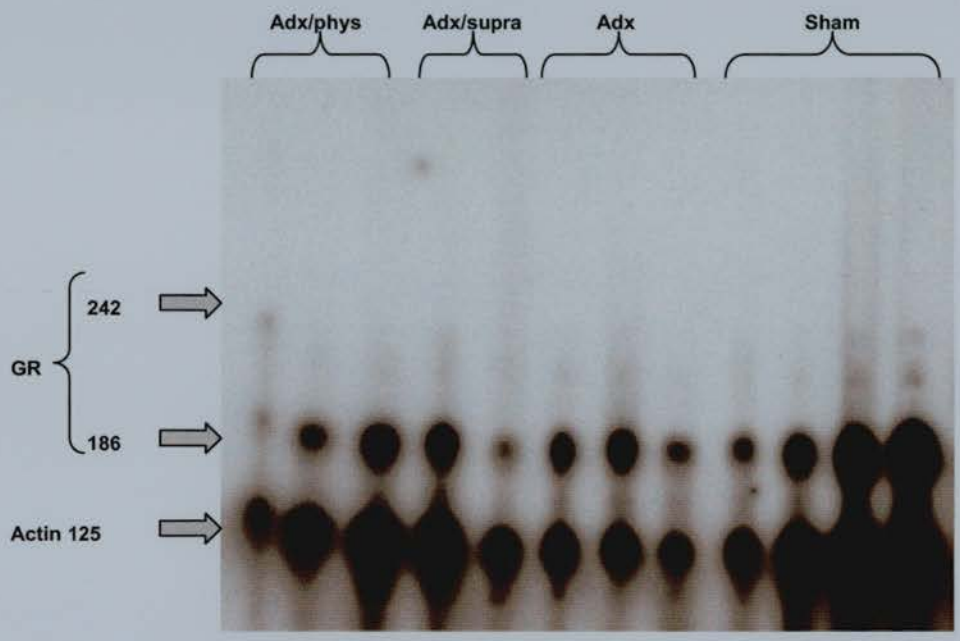


Figure 5.10: representative autoradiograph showing RNase protection analysis of GR mRNA containing exon 1_5 in livers of ST animals.

Each lane shows the product of an RPA reaction carried out on 50μg of liver RNA from a single animal. Arrows indicate the expected location of the 242 nucleotide protected fragment produced by GR mRNA containing both exon 1_5 and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_5 and the 125 nucleotide protected fragment produced by actin mRNA.

Autoradiograph shown is a 10d exposure.

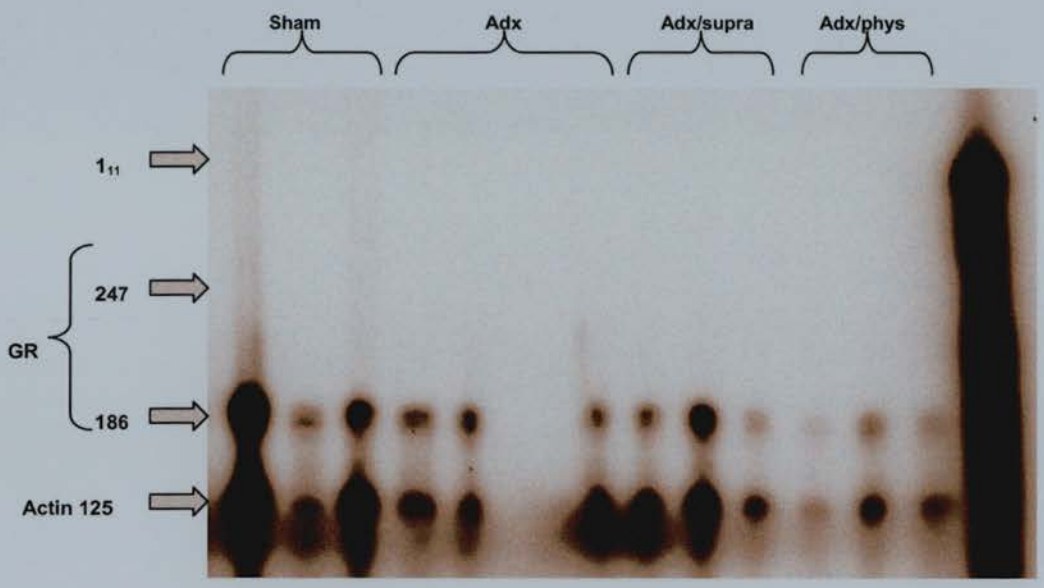


Figure 5.11: representative autoradiograph showing RNase protection analysis of GR mRNA containing exon 1_{11} in livers of ST animals.

Arrows indicate the full-length exon 1_{11} probe, the expected location of the 247 nucleotide protected fragment produced by GR mRNA containing exon 1_{11} and exon 2, the 186 nucleotide protected fragment produced by GR mRNA containing exon 2 but not exon 1_{11} and the 125bp protected fragment produced by actin mRNA.

Autoradiograph shown is a 7d exposure.

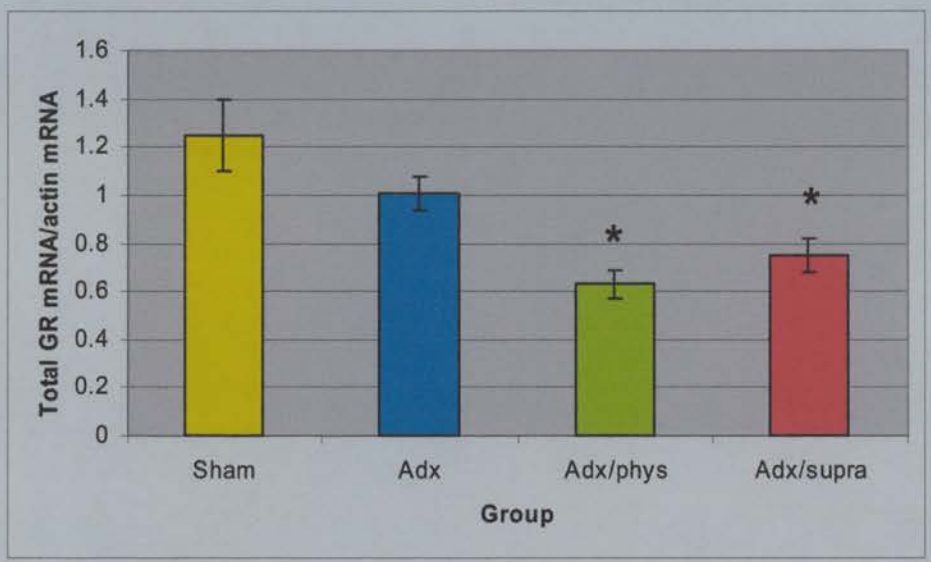


Figure 5.12: RNase protection analysis of total GR mRNA in liver of ST animals.

Data (presented as mean ± SEM) is compiled from 4 separate RNase protection analysis experiments (shown in Figure 5.7 and Figure 5.9 and represented in Figure 5.10 and Figure 5.11) using probes specific for exon 2 and one of exons 1_5, 1_6, 1_{10} and 1_{11} of the GR gene.

Ratios are calculated as described in section 2.2.11.3. Data from each experiment was normalised to the mean value for the Adx animals, then the values for each animal were averaged across all 4 experiments. Data were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR mRNA expression, which was significantly lower in the Adx/phys and Adx/supra animals than in the Sham animals ($p<0.05$, indicated by *).

n=6 per group except Sham where n=7.

5.3.3 *In situ* mRNA hybridization reveals that expression of total GR mRNA varies with glucocorticoid manipulations in ST but not LT animals.

An approximately 2x reduction in the total amount of GR mRNA containing exon 1_{10} was seen with glucocorticoid replacement in adrenalectomised animals, along with a near-significant approximately 2x reduction in the total amount of GR mRNA containing exon 1_6. Although highly quantitative, RNase protection analysis cannot provide anatomical resolution in a complex organ like the liver, so region-specific changes might be masked. In view of this, *in situ* mRNA hybridization was performed to determine whether there was a region-specific effect of treatment on GR mRNA expression. Since there was little change in GR mRNA levels in the brains of LT animals after glucocorticoid manipulation, the livers from these animals were not included in the RNase protection analysis. However, as there might be small but significant difference in the region-specific regulation of GR mRNA and variant exons 1 in these animals, their livers were included in the *in situ* mRNA hybridisation experiments.

There was clear zonal expression of total GR mRNA in the livers of both ST and LT animals (Figure 5.13), with expression appearing higher periportally in all animals (Figure 5.14). Densitometric analysis of autoradiographs of whole sections of liver tissue showed a significant effect of adrenalectomy with glucocorticoid replacement compared to adrenalectomy alone in the ST animals, in broad agreement with RNase protection analysis (Figure 5.15). However, there was no effect of glucocorticoid manipulations in the LT animals (Figure 5.15). To determine whether regulation of GR mRNA in the periportal and perivenous regions of ST animals was similar, GR mRNA expression in the periportal and perivenous regions was analysed by densitometry. This revealed significant changes in GR mRNA levels in both regions (Figure 5.16).

Densitometry gives a good assessment of the overall changes in mRNA expression in different regions of the tissue, but it does not provide cellular detail. To provide this level of resolution grain counting analysis of GR mRNA was performed on sections

of liver from both ST and LT animals. This showed a significant overall effect of treatment on GR mRNA in both periportal and perivenous regions in the ST animals, confirming the data from analysis of autoradiographs (Figure 5.17). In the periportal region, physiological glucocorticoid replacement caused a significant reduction in GR mRNA compared to sham-operated controls, while expression in both groups of animals receiving glucocorticoid replacement was significantly lower than in Adx animals which received no glucocorticoid treatment (Figure 5.17 A). The differences between groups in the perivenous region failed to reach statistical significance when analysed using Tukey's HSD Test (Figure 5.17 A). However, analysis using Fisher's LSD Test showed that physiological glucocorticoid replacement caused a significant reduction in GR mRNA compared to sham-operated controls, while expression in both groups of animals receiving glucocorticoid replacement was significantly lower than in Adx animals which received no glucocorticoid treatment (Figure 5.17 A).

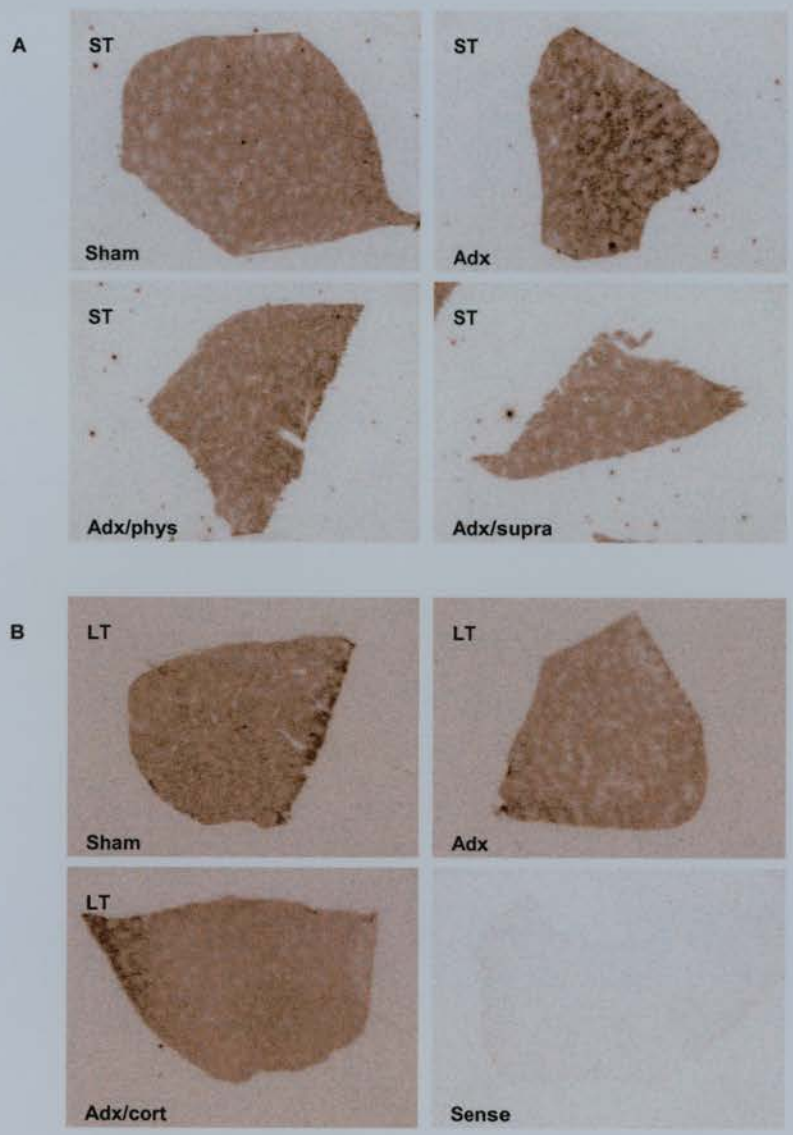
Interestingly, although glucocorticoid manipulation had no effect on total GR mRNA levels in livers of LT animals, the ratio of expression of GR in the periportal compared to the perivenous region of the hepatic acinus is altered by changes in glucocorticoid levels in LT animals (Figure 5.18). Adrenalectomy caused a significant increase in the periportal:perivenous ratio, an effect that was abolished by physiological corticosterone replacement (Figure 5.18). In contrast, ST animals showed no difference in the periportal:perivenous ratio of GR expression between the treatment groups.

Figure 5.13 (facing page): representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR cDNA to rat liver.

A: ST animals.

B: LT animals.

ST = short-term adrenalectomy, LT = long-term adrenalectomy, Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by injection, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet, Sense = sense control representative of both ST and LT animals.


A
ST
Sham
ST
Adx
ST
Adx/phys
ST
Adx/supra
B
LT
Sham
LT
Adx
LT
Adx/cort
Sense

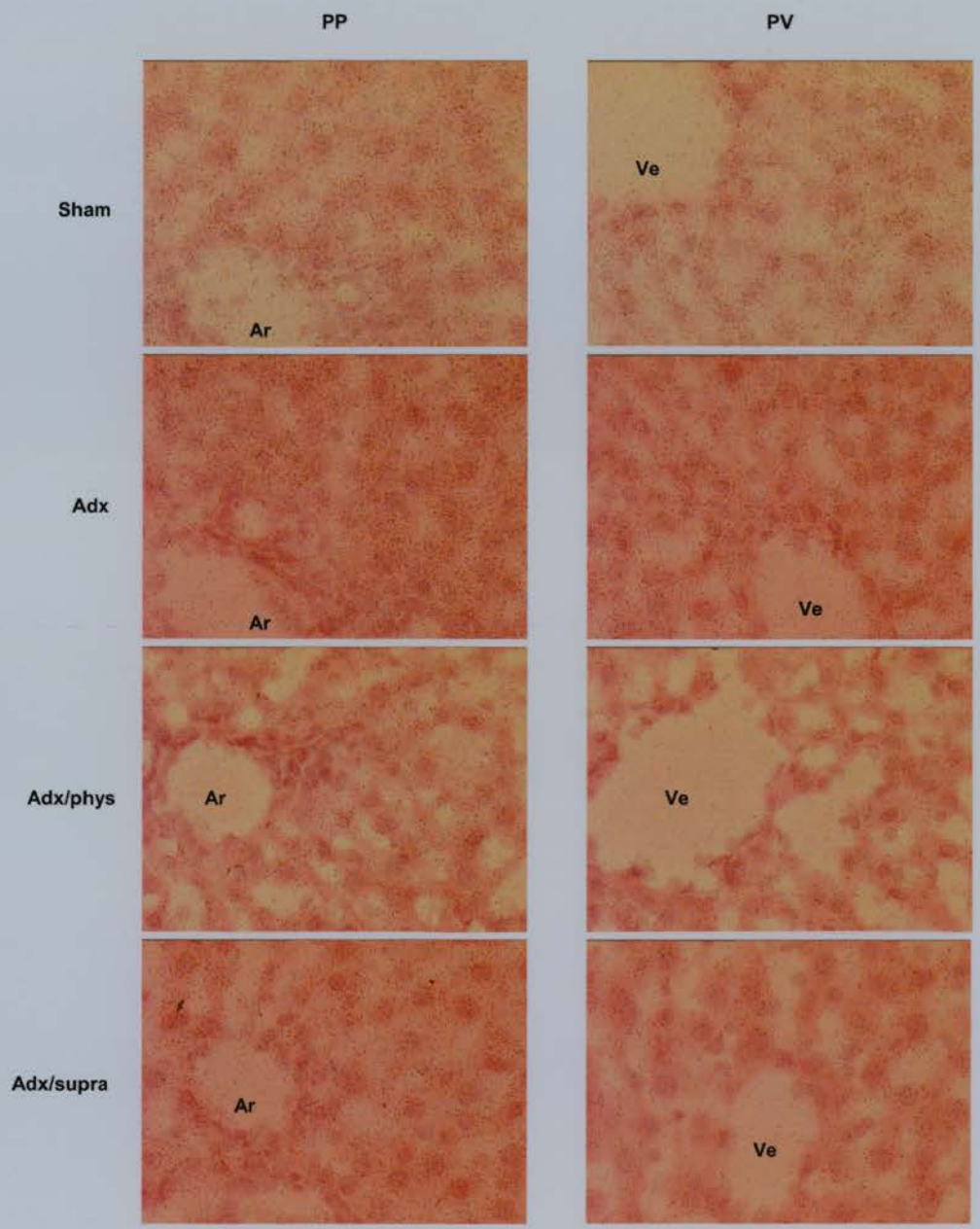


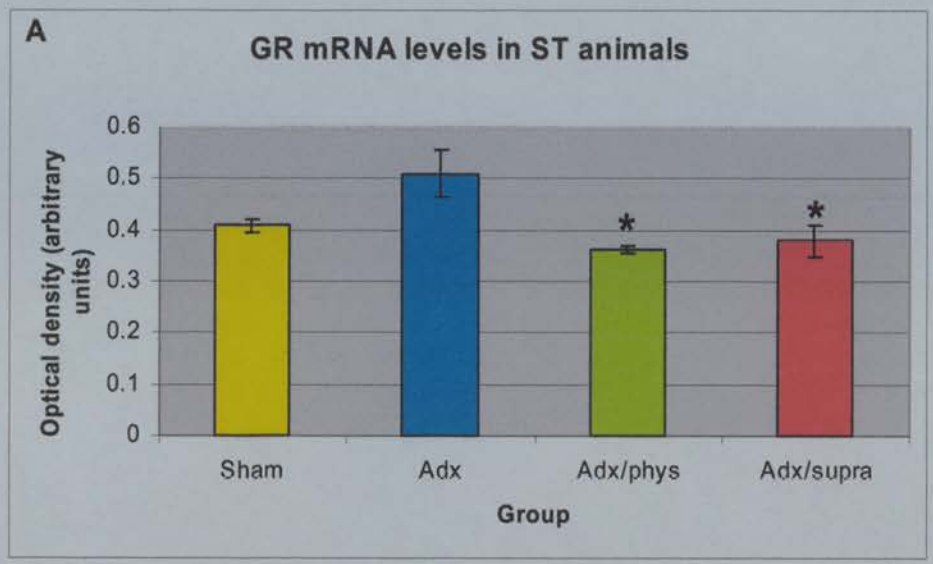
Figure 5.14: representative photomicrographs showing GR mRNA in periportal (PP) and perivenous (PV) regions of livers of ST animals.

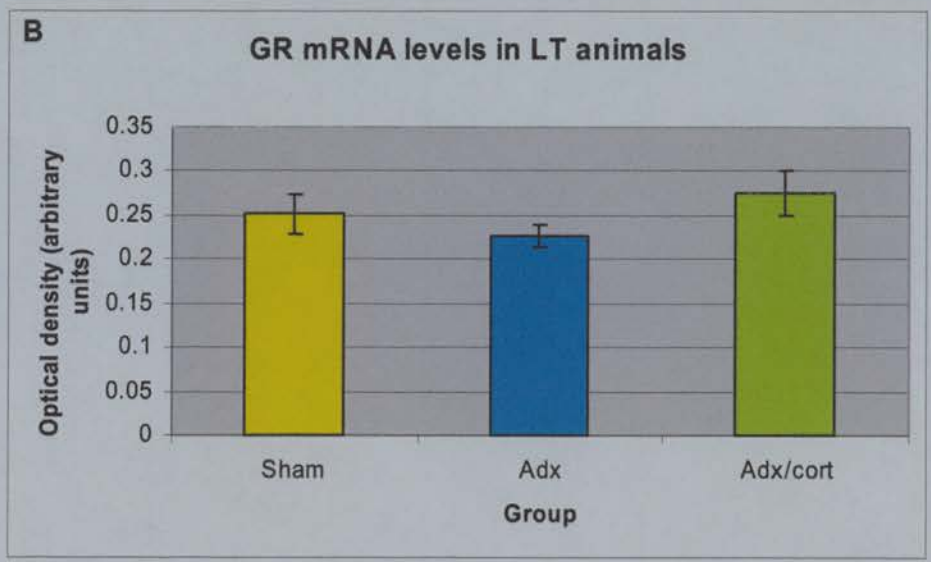
Magnification x 400. Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement, Ve = portal vein, Ar = hepatic arteriole.

Figure 5.15 (facing page): densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR cDNA to rat liver.

A: ST animals.D ata (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR mRNA expression. Although no group differed significantly from Sham animals, expression of GR in both Adx/phys and Adx/supra animals was significantly lower than that in Adx animals ($p<0.05$, denoted by *). n=6 per group except sham where n=7.

B: LT animals. ANOVA revealed no significant effect of treatment on GR expression. n=8 except Adx n=7.


A
GR mRNA levels in ST animals
Optical density (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Sham
Adx
Adx/phys
Adx/supra
Group


B
GR mRNA levels in LT animals
Optical density (arbitrary units)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Sham
Adx
Adx/cort
Group

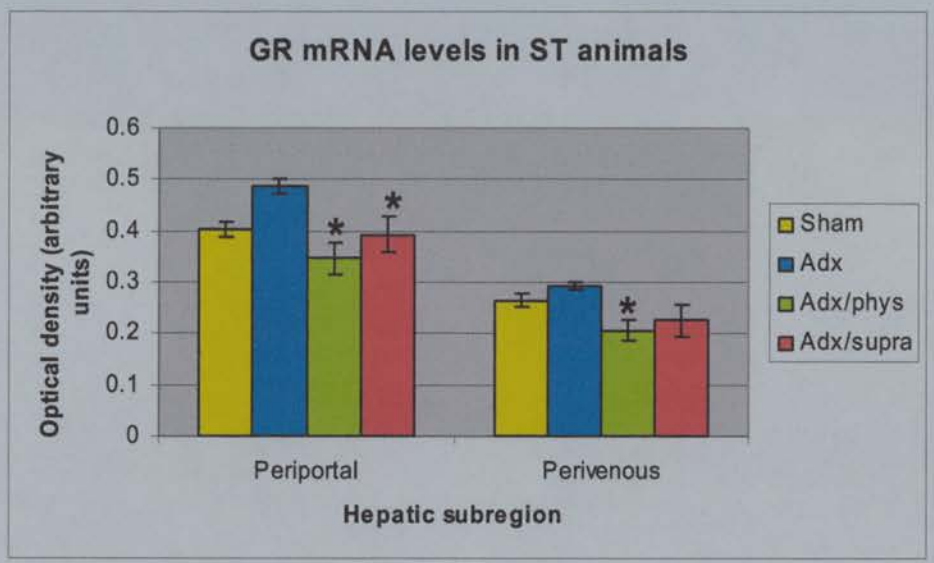


Figure 5.16: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the GR cDNA to livers of ST animals.

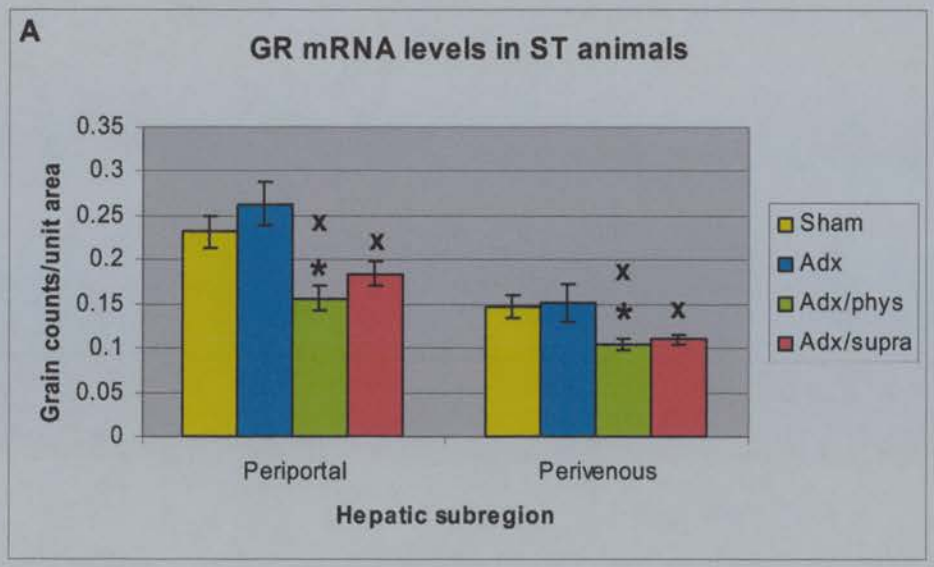
Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

There was a significant effect of treatment on GR mRNA expression in both periportal and perivenous regions. No group differed significantly from the Sham animals in either region. However, in the periportal region GR mRNA in both the Adx/phys and Adx/supra animals was significantly lower than in the Adx animals while in the perivenous region GR expression in the Adx/phys animals was significantly lower than in the Adx animals. Marked results (*) are significantly different to Adx animals ($p<0.05$). n=6 except Adx n=5 and Sham n=7.

Figure 5.17 (facing page): grain counting analysis of *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of the rat GR cDNA to rat liver.

A: ST animals.D ata (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR mRNA levels in periportal and perivenous regions. In the periportal region, expression in Adx/phys animals was significantly lower than in Sham animals ($p<0.05$: indicated by *) while expression in both Adx/phys and Adx/supra animals was significantly lower than in Adx animals ($p<0.05$: indicated by x).In the perivenous region, *post-hoc* analysis using Fisher's LSD Test showed that expression in Adx/phys animals was significantly lower than in Sham animals ($p<0.05$: indicated by *) while expression in both Adx/phys and Adx/supra animals was significantly lower than in Adx animals ($p<0.05$: indicated by x). n=6 except Adx n=5 and Sham n=7.

B: LT animals. ANOVA revealed no significant effect of treatment on GR expression in periportal or perivenous regions. n=8 except Adx n=5.


A
GR mRNA levels in ST animals
Grain counts/unit area
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Periportal
Perivenous
Hepatic subregion
Sham
Adx
Adx/phys
Adx/supra

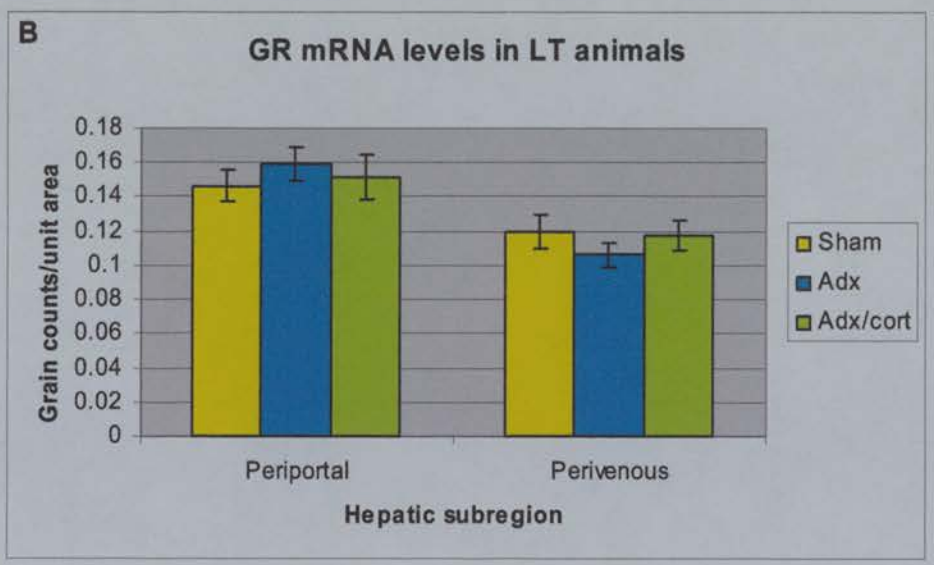
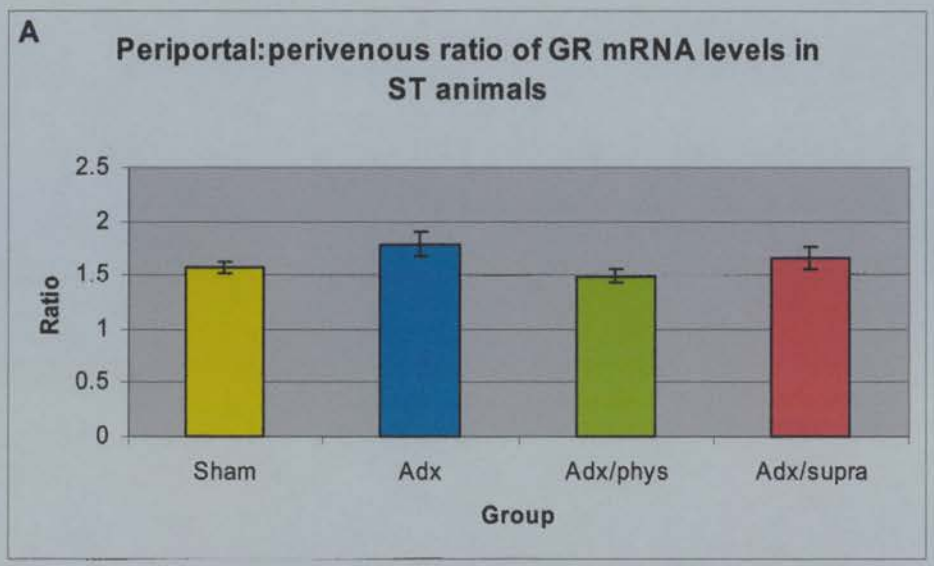
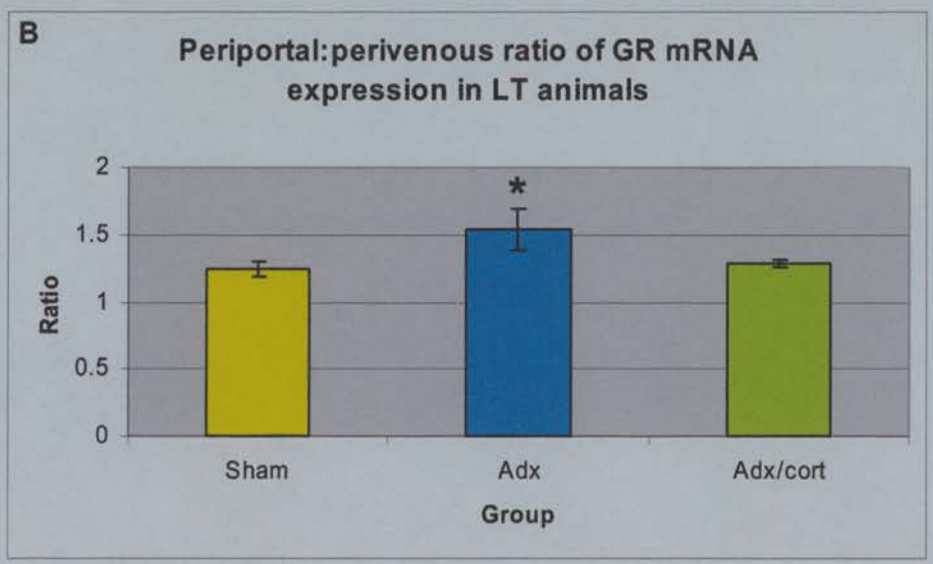

B
GR mRNA levels in LT animals
Grain counts/unit area
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Periportal
Perivenous
Hepatic subregion
Sham
Adx
Adx/cort

Figure 5.18 (facing page): grain counting analysis of periportal:perivenous ratio of GR mRNA expression in rat liver.

A: ST animals. Data are presented as mean ± SEM.A NOVA showed no significant effect of treatment on periportal:perivenous ratio. n=6 except Adx n=5 and Sham n=8.

B: LT animals. Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on periportal:perivenous ratio, which was significantly higher in Adx animals than in Sham animals (p=0.05, indicated by *). n=8 except Adx n=5.


A
Periportal:perivenous ratio of GR mRNA levels in ST animals
2.5
2
1.5
1
0.5
0
Ratio
Sham
Adx
Adx/phys
Adx/supra
Group


B
Periportal:perivenous ratio of GR mRNA expression in LT animals
2
1.5
1
0.5
0
Ratio
*
Sham
Adx
Adx/cort
Group

5.3.4 *In situ* mRNA hybridization analysis of the distribution and regulation of GR mRNA transcripts containing variant exons 1 in the liver.

In situ mRNA hybridisation had revealed zonal distribution and glucocorticoid-induced changes in total GR mRNA levels in livers of ST animals, as well as a change in the periportal:perivenous ratio of GR mRNA in LT animals. Therefore the same technique was used to investigate the distribution of GR mRNA containing variant exons 1 in the livers of ST and LT animals and the effects of glucocorticoid manipulation on their expression.

There was zonal expression of exon 1_{10} in the livers of ST and LT animals (Figure 5.19). Densitometric (Figure 5.20) and grain counting (Figure 5.21, Figure 5.22) analysis showed no effect of glucocorticoid manipulation on expression of GR mRNA containing exon 1_{10} in ST or LT animals. In addition, the periportal:perivenous ratio of GR mRNA containing exon 1_{10} was unaffected by glucocorticoid manipulations in ST and LT animals, although there was a strong trend for treatment to affect the periportal:perivenous ratio in the LT animals ($p=0.055$, Figure 5.23), similar to that seen for total GR mRNA.

In agreement with the RPA results, *in situ* mRNA hybridisation using a probe specific for exon 1_{11} showed no specific hybridisation (data not shown).

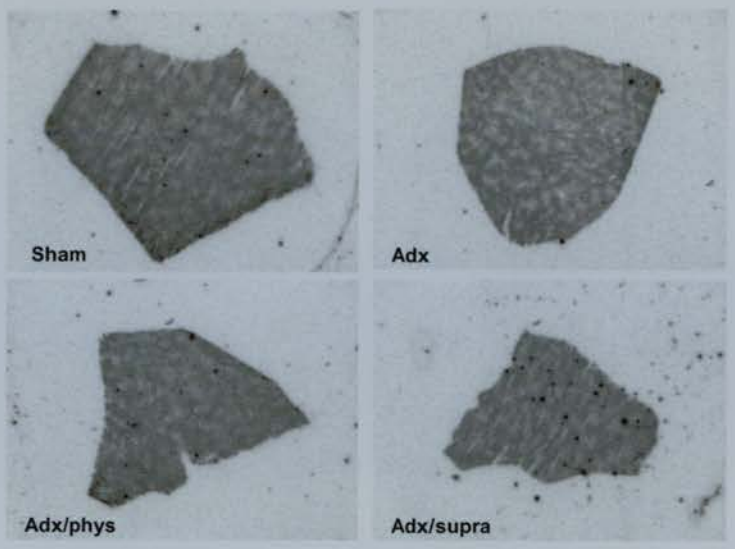
Figure 5.19 (facing page): representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to rat liver.

A: ST animals

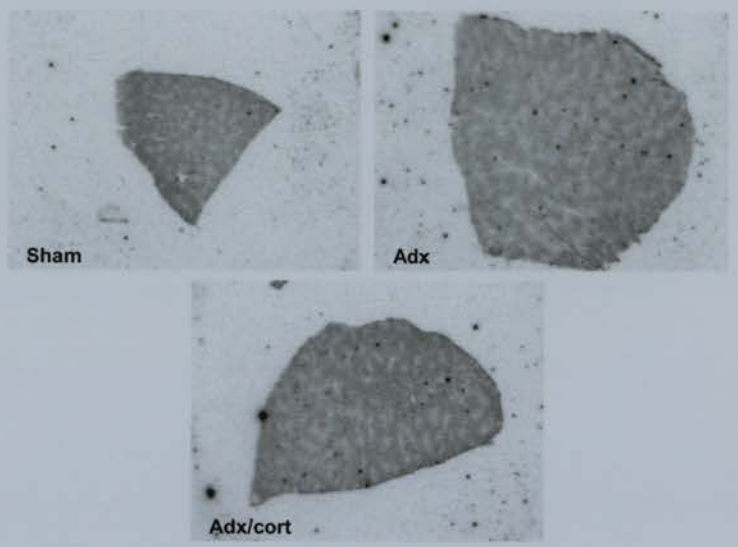
B: LT animals

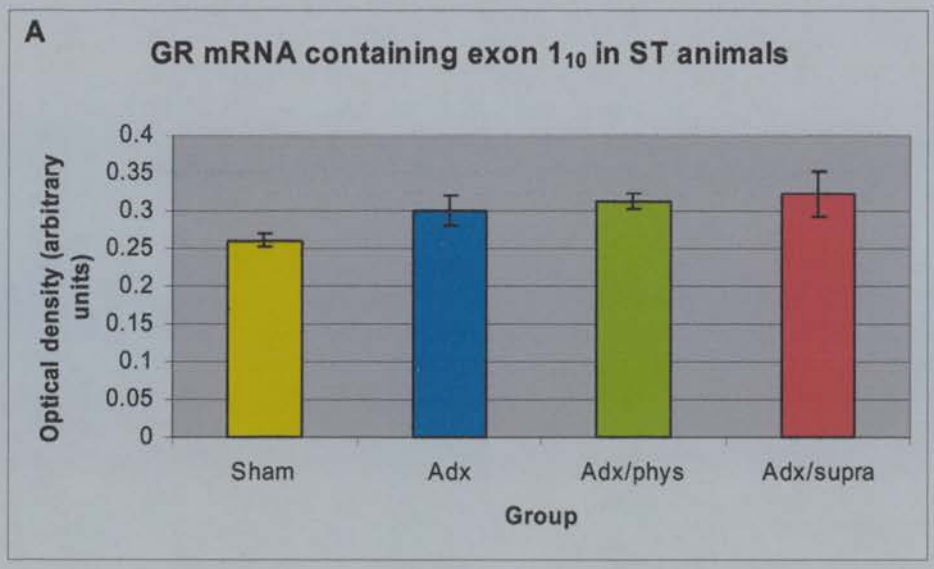
ST = short-term adrenalectomy, LT = long-term adrenalectomy, Sham = sham-operated control, Adx = adrenalectomised, Adx/phys = adrenalectomised with physiological corticosterone replacement by injection, Adx/supra = adrenalectomised with supraphysiological corticosterone replacement by injection, Adx/cort = adrenalectomised with physiological corticosterone replacement by subcutaneous pellet, Sense = sense control representative of both ST and LT animals.

A



B





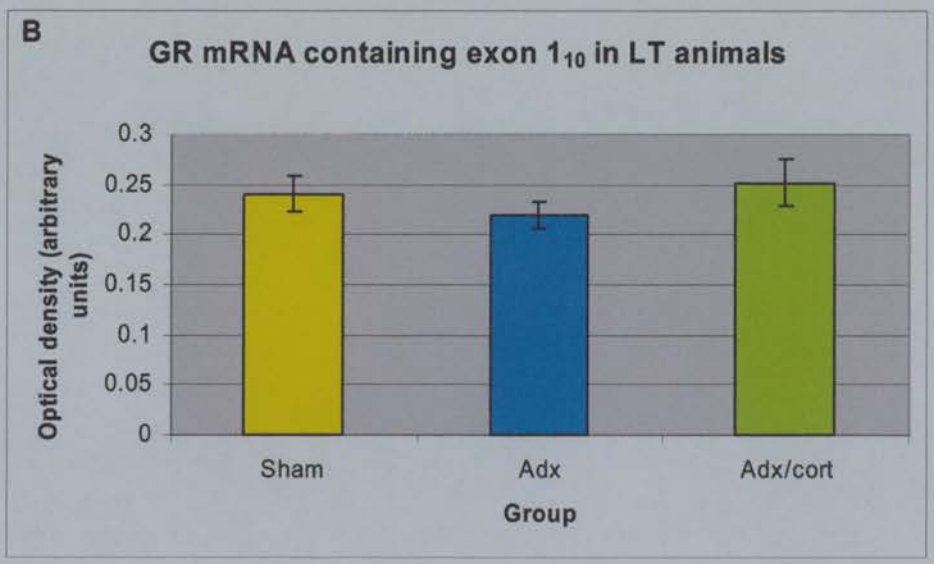


Figure 5.20: densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to rat liver.

Data are presented as mean ± SEM

A: ST animals. ANOVA showed no significant effect of treatment on exon 1_{10} expression.

B: LT animals. ANOVA showed no significant effect of treatment on exon 1_{10} expression.

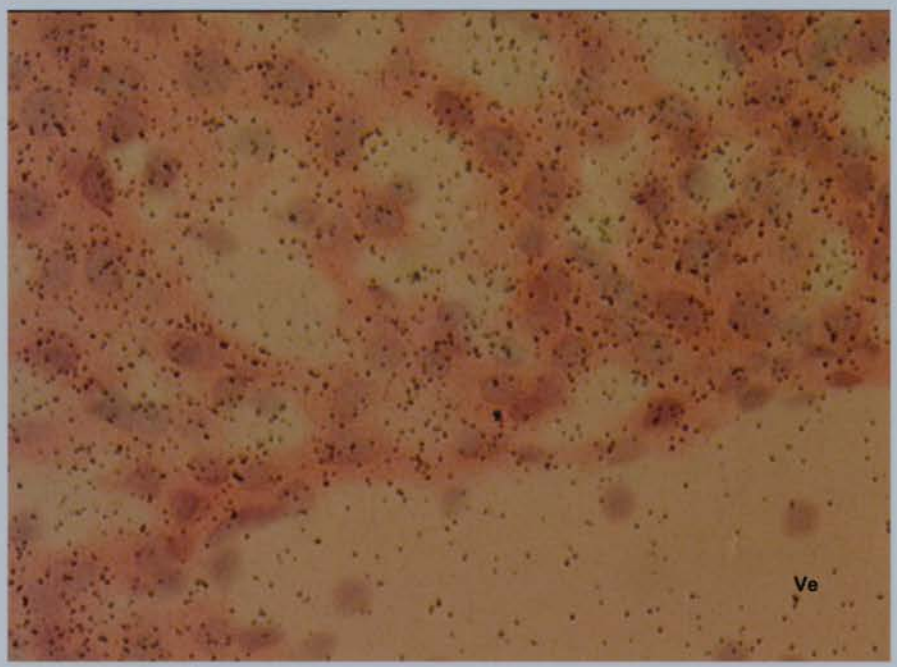
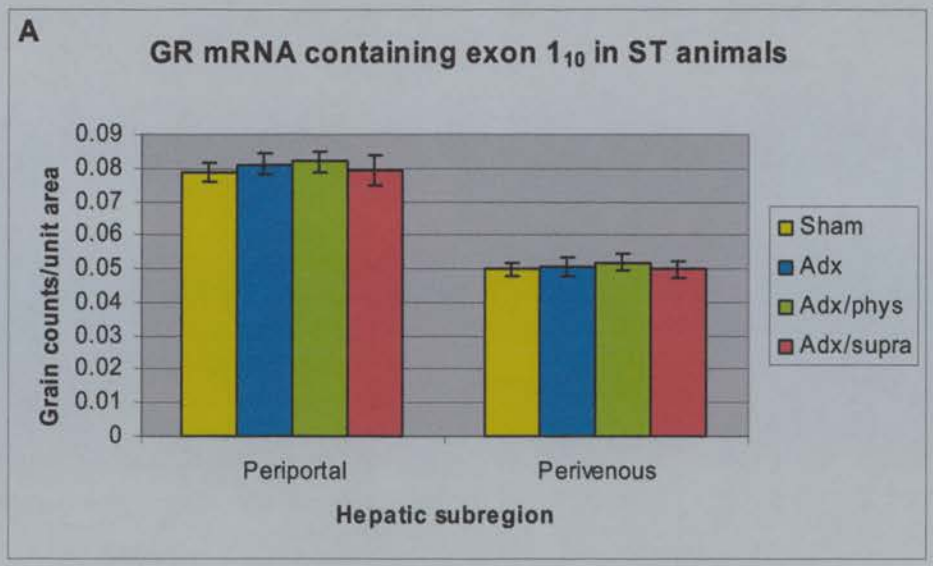


Figure 5.21: representative photomicrograph showing silver grains after *in situ* mRNA hybridisation of a probe specific for exon 1_{10} of the GR gene to ST rat liver.

Magnification x 400. Ve = portal venule.



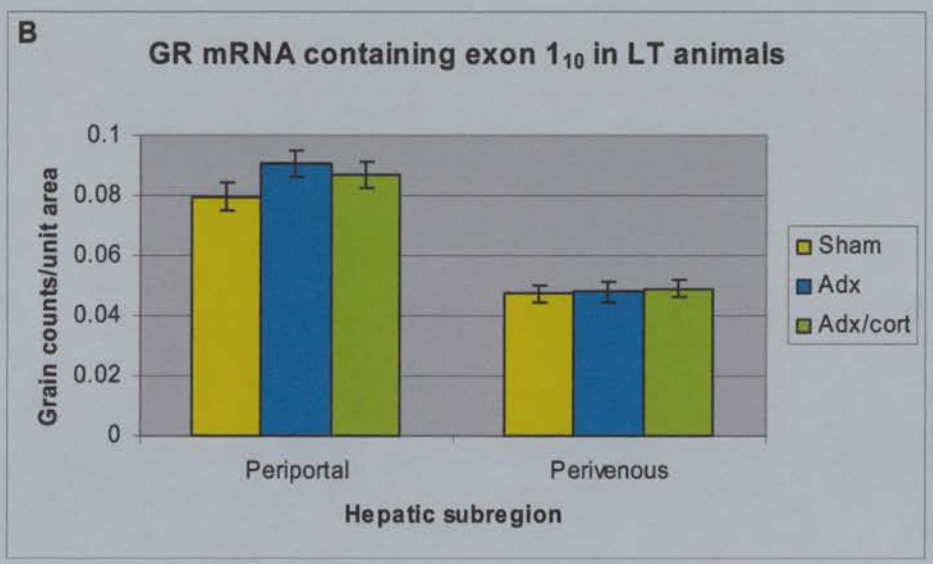


Figure 5.22: grain counting analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to rat liver.

A: ST animals. Adx/phys n=6, Adx/supra n=4, Adx n=5 and Sham n=7.

B: LT animals. n=8 except Adx n=6.

ANOVA showed no significant effect of treatment on exon 1_{10} expression in periportal or perivenous regions for either ST or LT animals.

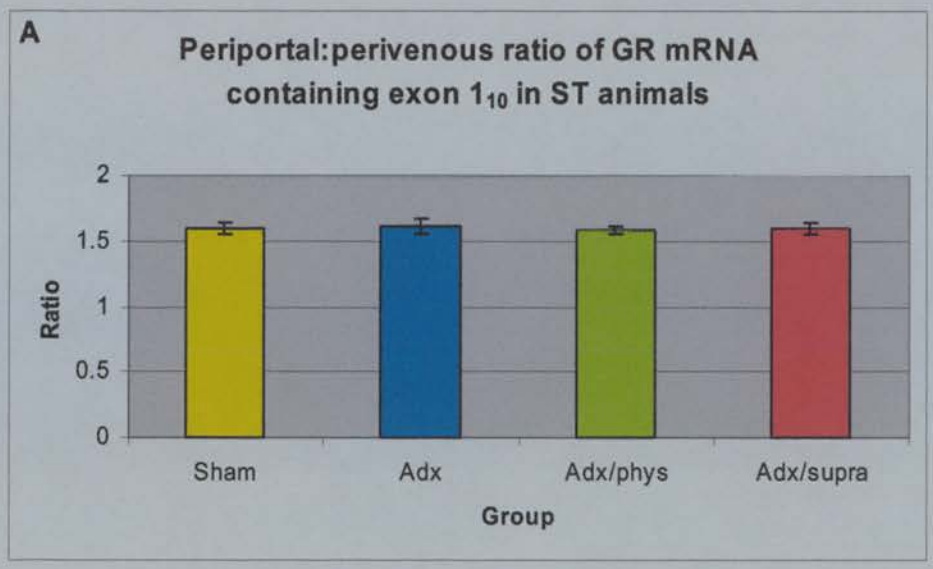
Figure 5.23 (facing page): analysis of periportal:perivenous ratio of GR mRNA containing exon 1_{10}, determined by grain counting.

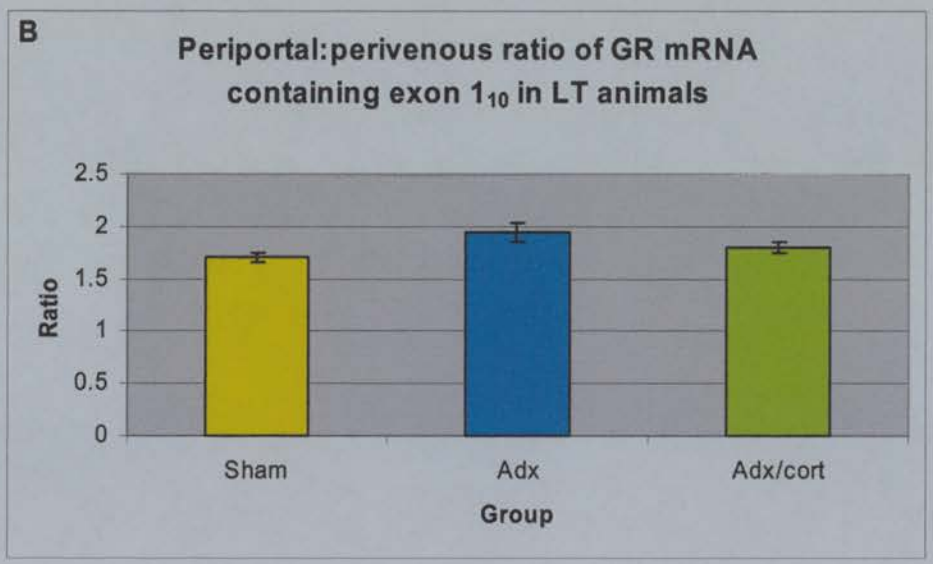
A: ST animals. Adx/phys n=6, Adx/supra n=4, Adx n=5 and Sham n=7.

B: LT animals. n=8 except Adx n=6.

Data are presented as mean ± SEM.

ANOVA showed no significant effect of treatment on periportal:perivenous ratio of GR mRNA containing exon 1_{10} in either experiment.


A
Periportal:perivenous ratio of GR mRNA containing exon 1$_{10}$ in ST animals
Ratio
2
1.5
1
0.5
0
Sham
Adx
Adx/phys
Adx/supra
Group


B
Periportal:perivenous ratio of GR mRNA containing exon 1$_{10}$ in LT animals
Ratio
2.5
2
1.5
1
0.5
0
Sham
Adx
Adx/cort
Group

5.3.5 Preliminary data reveals a specific area of DNase I sensitivity in the GR gene promoter region.

To determine if tissue-specific differential expression of exons 1 was associated with changes in the chromatin structure of the GR gene promoter region, preliminary experiments were performed to localise DNase I hypersensitive sites in liver chromatin at the 5' end of the GR gene. The material used was obtained from control Wistar rats.

The location of restriction enzyme sites and probe used are shown in Figure 5.24. A *NcoI* fragment spanning nucleotides -4534 to -2350 of the GR gene, which includes exons 1_4 to 1_9 and part of exon 1_{10} (Figure 5.24), was sensitive to DNase I digestion (Figure 5.25). However, no smaller "daughter" band was observed, suggesting that this may not be a true DNase I hypersensitive site but rather a region of general DNase I sensitivity. In contrast, the *PstI* fragment extending from ≈ -5000 to –3326 and the *PvuII* fragment extending from ≈ -5300 to -3911 (Figure 5.25) showed little or no DNase I sensitivity.

To localise the region of DNase sensitivity the NcoI fragment was cleaved in double digests carried out on the same DNA samples. None of the fragments were affected by DNase I (Figure 5.26). This suggests that the observed region of DNase I sensitivity lies between –2533 and -2350.

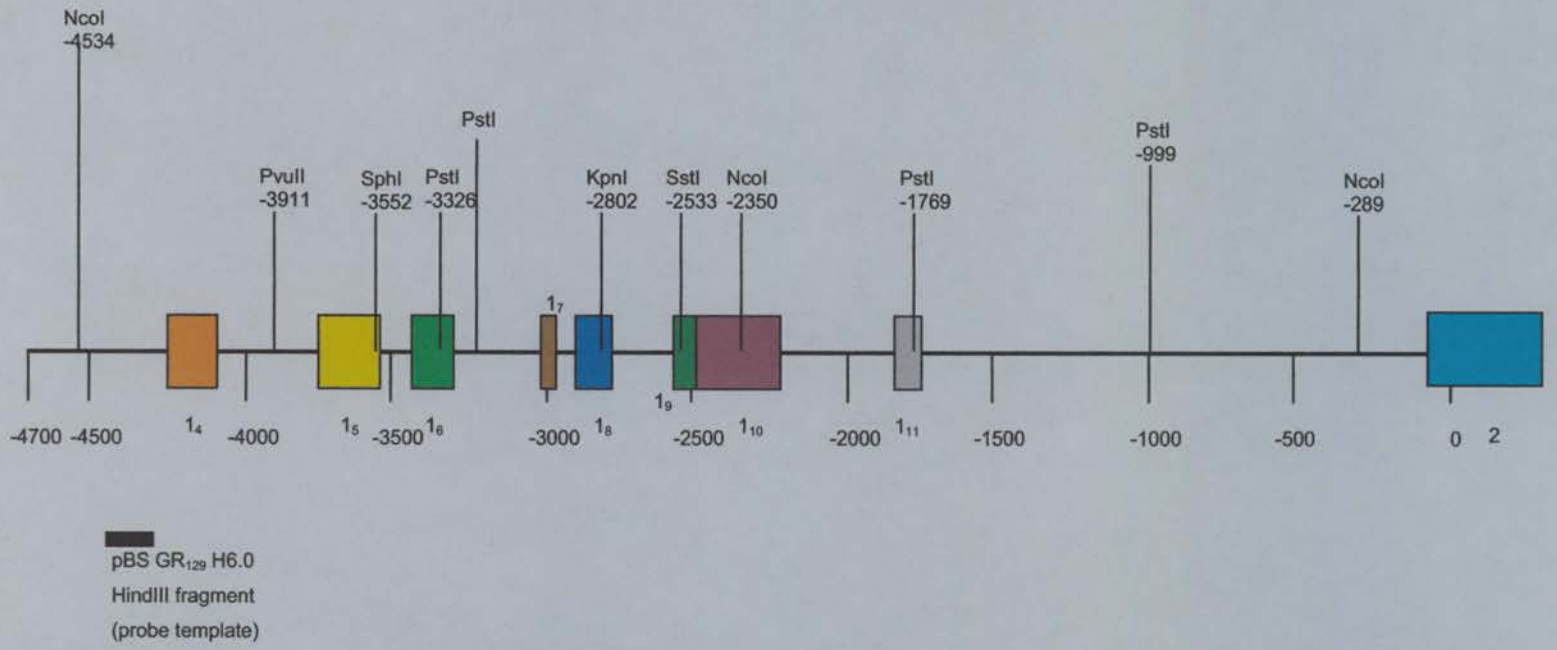


Figure 5.24: restriction map of the 5' end of the GR gene. Coloured boxes indicate the positions of variant exons 1. Numbering is relative to the translation start site within exon 2. Relevant restriction enzyme sites are marked. The black bar indicates the probe used in Southern analysis.

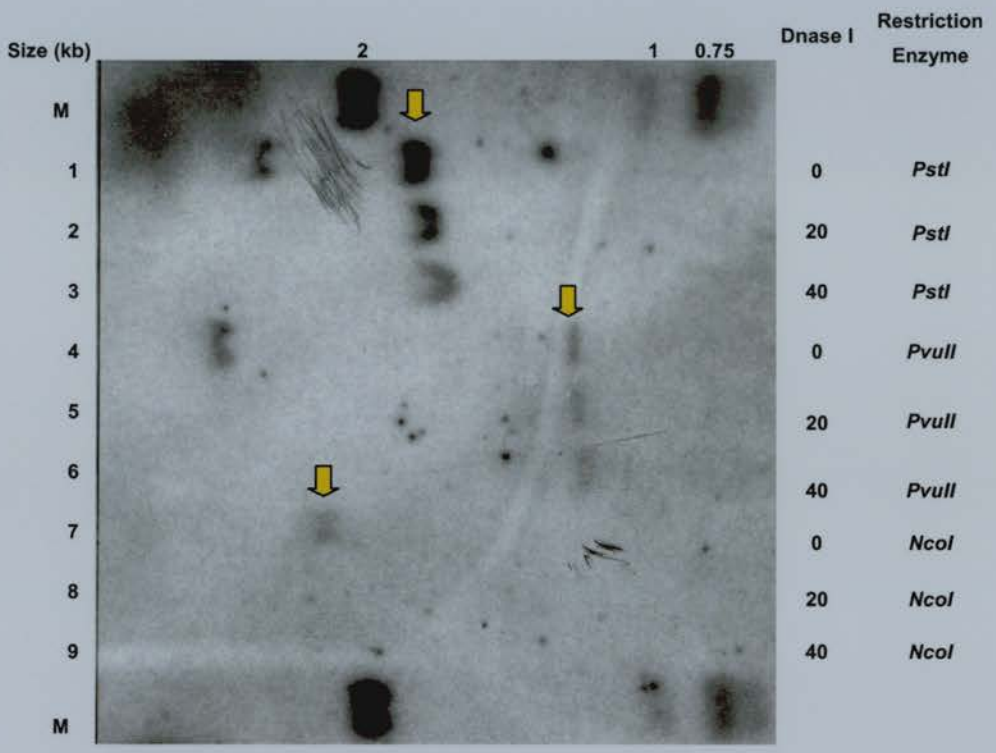


Figure 5.25: representative autoradiograph showing DNase I hypersensitive site mapping of rat liver genomic DNA.

Nuclei were treated with 0, 20 or 40 units of DNase I, DNA was extracted, digested with *PstI*, *PvuII* or *NcoI* then blotted as described in section 2.2.8 and hybridised to radiolabelled probe corresponding to -4438 to -4300 (see Figure 5.24). Autoradiograph was exposed for 12hr.

Arrows indicate hybridising fragments: the 1.7kb *PstI* fragment (≈ -5000 to -3326), the 1.4kb *PvuII* fragment (≈ -5300 to -3911) and the 2.2kb *NcoI* fragment (-4534 to -2350). The *PstI* and *PvuII* fragments are unaffected by DNase I treatment while the *NcoI* fragment is DNase I sensitive.

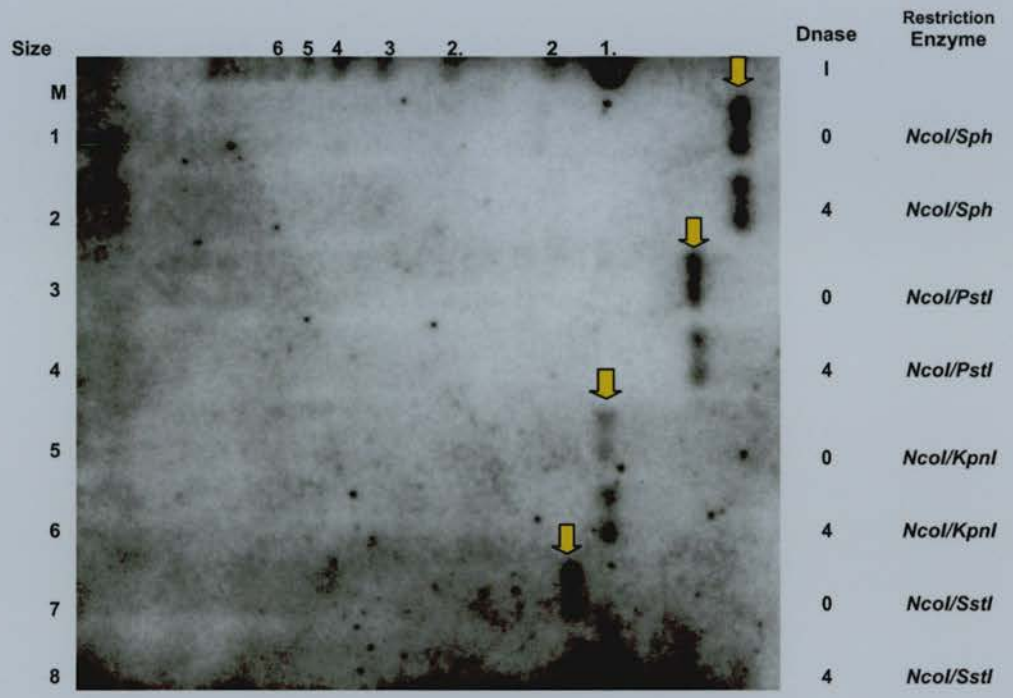


Figure 5.26: representative autoradiograph showing localisation of a region of DNase sensitivity in rat liver genomic DNA.

Nuclei were treated with 0 or 40 units of DNase I, DNA was extracted, digested with *NcoI* together with either *SphI*, *PstI*, *KpnI* or *SstI* then blotted as described in section 2.2.8 and hybridised to a probe generated from the template shown in Figure 5.24. Autoradiograph was exposed for 12hr.

Arrows indicate hybridising fragments: the ≈0.9kb *NcoI/SphI* fragment (-4534 to -3552), the 1.1kb *NcoI/PstI* fragment (-4534 to -3362), the 1.6kb *NcoI/KpnI* fragment (-4534 to -2802) and the 1.9kb *NcoI/SstI* fragment (-4534 to -2533). None of the fragments are affected by DNase I treatment.

5.4 Discussion

Consistent with previous data (McCormick et al., 2000), exon 1_{10} is present in the majority (≈ 63%) of GR mRNA in liver, with a substantial minority (≈ 14%) containing exon 1_6. These exons together are therefore present in only approximately 77% of the GR mRNA in liver. A small amount of GR mRNA containing exons 1_5 and 1_{11} was previously detected in liver (McCormick et al., 2000), although they were undetectable in these experiments. Thus, ≈23% of GR mRNA remains unaccounted for, suggesting that other (perhaps novel) exons 1 may be expressed in liver. This is consistent with previous data (McCormick et al., 2000).

RNase protection analysis demonstrated glucocorticoid regulation of GR mRNA containing exon 1_{10} and a strong trend for glucocorticoid manipulation to regulate the expression of GR mRNA containing exon 1_6. In both cases the magnitude and direction of these changes is very similar to the changes seen in total GR mRNA analysed by both RNase protection assay and *in situ* mRNA hybridisation. However, *in situ* mRNA hybridisation failed to demonstrate any effect of treatment on exon 1_{10} expression, possibly because it is less quantitative than RNase protection. Exons 1_6 and 1_{10} together are present in the majority of GR mRNA in the liver (this work and (McCormick et al., 2000)), suggesting that the observed changes in the total levels of these transcripts reflect the changes in total GR mRNA levels caused by glucocorticoid manipulation. Conversely, exons 1_5 and 1_{11} were undetectable by RNase protection analysis in these experiments, thus even if GR mRNA containing these exons was subject to glucocorticoid regulation they would make only a very small contribution to any change in total GR mRNA levels. It remains possible, however, that expression of GR mRNA containing novel exon 1 variants is being regulated by glucocorticoid manipulation.

There was a strong trend for changes in glucocorticoid levels to affect the proportion of the total GR mRNA containing exon 1_6 (determined by RNase protection analysis). Since exon 1_6 forms only ≈ 14% of total GR mRNA in liver, the statistical effect of experimental variation will be greater than for exon 1_{10}, which is present in ≈ 63% of GR mRNA. This may explain why these data failed to reach statistical

significance. Alternatively, expression of exon 1_6 might be changing in one part of the hepatic acinus but not the other, with the change being partially masked since the RNase protection analysis used RNA derived from homogenised liver. It was not possible to carry out *in situ* mRNA hybridisation for exon 1_6 as the region containing 1_6 is short and extremely GC-rich, so an appropriate probe template could not be designed (discussed in Chapter 3). Repeating the RNase protection analysis using RNA obtained from separated periportal and perivenous hepatocytes would address this question. However, it is possible that expression of exon 1_6 is unaffected by changes in glucocorticoid levels.

RNase protection analysis cannot detect region-specific changes in mRNA levels in a complex organ such as the liver. *In situ* mRNA hybridisation was used to investigate the anatomical distribution of total GR mRNA as well as GR mRNA containing exon 1_{10} and to determine whether levels of these transcripts changed in a particular region of the hepatic acinus. There was clear differential expression of GR mRNA in the periportal and perivenous regions of the hepatic acinus, as previously described (Nyirenda et al., 1998) and noted in Chapter 3. Also, there was clear zonal expression of GR mRNA containing exon 1_{10}, with a periportal:perivenous ratio similar to that of total GR mRNA. Interestingly, the distribution of GR protein has been previously described as homogeneous in the liver (Antakly and Eisen, 1984), although this report did not quantitatively assess GR levels in the periportal and perivenous hepatocytes. Also, there may be a difference between GR mRNA and protein levels in a tissue since there is some post-transcriptional regulation of GR levels (Dong et al., 1988; Paskitti et al., 2000). The differentiation of hepatic metabolism in the periportal and perivenous hepatocytes (the "metabolic zonation" theory) and the distribution of enzymes regulated by glucocorticoids is discussed in detail in section 1.9.4. It is possible that GR is co-localised periportally with the enzymes of gluconeogenesis, where a higher level of GR will confer greater glucocorticoid sensitivity.

In contrast to the observed effect in the hippocampus (Chapter 4) adrenalectomy did not significantly increase hepatic total GR mRNA levels compared to controls in either the periportal or perivenous regions. This is in agreement with previous data,

where 2 week adrenalectomised rats showed only a 15% increase in hepatic GR compared to a 40% increase in brain (Kalinyak et al., 1987). Interestingly, there may be species-specific differences in the effect of adrenalectomy since 24h adrenalectomy in mice caused a 2-2.5 x increase in hepatic glucocorticoid binding capacity (Svec et al., 1989). However, it is possible that there is an acute change in hepatic GR with adrenalectomy that has returned to normal by the time the tissues from the ST and LT animals were harvested: this possibility is discussed further below. Alternatively, it may be that the increase in hepatic GR seen by Svec *et al* was not due to increased GR mRNA synthesis, but to a change in stability or translation efficiency of the GR mRNA or decreased rate of receptor degradation (none of which were assessed in the current study). This is possible, since there may be some post- transcriptional control of GR level by glucocorticoids (Dong et al., 1988; Paskitti et al., 2000).

In situ mRNA hybridisation showed that adrenalectomy and corticosterone replacement in ST animals significantly downregulated hepatic GR mRNA relative to adrenalectomy alone. This data is in agreement with previous studies where corticosterone treatment of adrenalectomised rats significantly reduced hepatic glucocorticoid binding capacity (Alexandrova et al., 1989), dexamethasone treatment of adrenal-intact rats caused a 40% decrease in hepatic GR mRNA levels (Kalinyak et al., 1987) and intraperitoneal injection of adrenalectomised mice with 1mg of corticosterone downregulated hepatic GR number by 25% compared to adrenalectomised animals (Svec, 1988). Also, rats subjected to repeated stressors showed significantly reduced hepatic GR number, attributed by the authors to sustained corticosterone secretion (Alexandrova and Farkas, 1992). However, the role of stress in regulation of hepatic GR is not clear; in adrenally-intact mice, 3d of repeated stressors did not affect glucocorticoid binding capacity in liver cytosol (Svec et al., 1989). This may be a species difference between rat and mouse: alternatively, it may be due to the tissues being obtained from the rats immediately post-stress (Alexandrova and Farkas, 1992) while those from the mice were obtained 24h after the last stressor (Svec et al., 1989).

In contrast to the *in situ* mRNA hybridisation results, RNase protection analysis showed that adrenalectomy and both doses of corticosterone replacement in ST animals significantly downregulated hepatic GR mRNA relative to sham-operated controls rather than to adrenalectomised animals. This difference is probably a statistical effect due to the RNase protection data being averaged from 4 separate experiments. Alternatively, it may be due to the difference in quantitative precision of the two techniques.

There was no difference in effect between the physiological and supraphysiological replacement doses of corticosterone, unlike in the hippocampus (Chapter 4). Also, neither adrenalectomy nor glucocorticoid replacement in LT animals produced a significant difference in hepatic GR mRNA compared to sham-operated controls. These data suggest that there are tissue-specific differences in GR regulation by glucocorticoids between hippocampus and liver.

An obvious question is why adrenalectomy or adrenalectomy with supraphysiological glucocorticoid replacement had no effect on hepatic GR levels compared to sham-operated controls. Since the LT animals showed no differences in total GR mRNA levels, perhaps there is an acute change in hepatic GR number with glucocorticoid manipulation that returns to the programmed "set-point" over time, as is seen in the hippocampus (Holmes et al., 1995b; Reul et al., 1989). It is possible that this was occurring by the time the tissues were harvested from the ST animals, resulting in a significant difference between the animals with the highest GR mRNA levels (the adrenalectomised group) and those with the lowest (the physiologically-replaced group) but no differences between the treatment groups and the sham-operated controls.

Alternatively, it is tempting to speculate that glucocorticoids have an important role in determining the "set point" of hepatic GR expression during development (where hepatic GR mRNA levels may be permanently "programmed" by prenatal exposure to dexamethasone (Nyirenda et al., 1998)) but do not dramatically regulate hepatic GR levels in the adrenally-intact adult animal. This would be logical in terms of the central role of GR in the control of hepatic metabolism (section 1.9.4). For example,

in an animal under chronic stress increased glucocorticoid levels would cause an increased glucocorticoid signal to the hepatocytes. However, if this signal caused the downregulation of hepatic GR the maximum level of glucocorticoid signal that could be transduced might eventually be reduced, impairing the liver's capacity to produce glucose during the fight or flight response by reducing the level of expression of gluconeogenic enzymes. This might plausibly put the animal at a disadvantage.

The increase in the periportal:perivenous ratio of total GR mRNA seen with adrenalectomy in the LT animals was unexpected. Adrenalectomy had no effect on total GR mRNA in either region individually and no similar change in ratio was seen in the ST animals despite the significant regional changes in GR mRNA. Possibly in the LT animals adrenalectomy increased GR mRNA slightly in the periportal region while decreasing it slightly in the perivenous, with neither change being significant alone but reaching significance when taken together. Indeed, GR mRNA in adrenalectomised animals tends to be higher in the periportal region and lower in the perivenous region than that of sham controls, but the individual differences were not significant. A change in the periportal:perivenous ratio of GR expression might be of relevance to the metabolic zonation theory discussed above and in Chapter 1. Further investigation of this effect with a larger number of animals to give greater statistical power is perhaps warranted.

Preliminary data is presented here regarding the chromatin structure of the GR gene. Technical difficulties and time limitations precluded a more detailed analysis. Although very preliminary, the results of these experiments in control animals suggest that a region of DNase I sensitivity lies close to or within exon 1_{10}. This would be consistent with the chromatin being open in this transcriptionally active area. The absence of the expected "daughter" bands from the blot may be due to the fact that these bands are often indistinct due to there being up to 200bp variability in the location of the site where DNase I cleaves the DNA . It could be argued that poor transfer of DNA onto the nylon membrane caused the disappearance of the *NcoI* fragment. This is unlikely, however, as the parent fragment as well as smaller fragments were efficiently transferred. Work is currently underway in our laboratory

to further characterise the chromatin structure of the GR promoter region in liver and a variety of cell lines.

The data presented here and in Chapter 4 have suggested that in hippocampus and liver variant exons 1 of the GR gene are regulated similarly to total GR mRNA. The results of experiments investigating the effects of glucocorticoids on the expression of GR and its alternate exons 1 in the thymus are described in the following chapter.

6 The effect of glucocorticoids on expression of the GR gene and alternate exons 1 in the thymus

6.1 Introduction

Glucocorticoids have an important role in the physiology of the immune system (section 1.9.5). The thymus has perhaps the highest level of GR in the body (Lowy, 1989; Reul et al., 1989). GR is down-regulated as T cells mature (Miller et al., 1998) and the amount of GR in tissues of the immune system parallels their sensitivity to glucocorticoids (Miller et al., 1998). Glucocorticoid downregulation of GR in the thymus and T lymphocytes is well-recognised (Miller et al., 1990; Peiffer et al., 1994; Spencer et al., 1991).

However, in some lymphoid cell lines glucocorticoids upregulate GR (Antakly et al., 1989; Antakly et al., 1990; Ashraf et al., 1991; Denton et al., 1993; Eisen et al., 1988). Also, there is evidence that, in human CEM-C7 cells, glucocorticoids increase expression of GR mRNA containing exon 1A (equivalent to rat exon 1_1) (Breslin et al., 2001). Current data suggests that expression of exon 1_1 is restricted to tissues of the immune system ((McCormick et al., 2000; Strahle et al., 1992), Chapter 3): indeed, exon 1A is the predominant exon 1 in GR mRNA from S49 lymphoma cells (Strahle et al., 1992). Furthermore, exon 1_1 is located at least 15kb 5' of exon 2 and most of the other known exons 1, which lie in a 3kb CpG island ≈1.5kb 5' of exon 2 (McCormick et al., 2000). These observations led to the hypothesis that expression of transcripts containing exon 1_1 might be regulated differently by glucocorticoids to that of the other exons 1. The experiments described in this chapter were designed to test this hypothesis.

6.2 Experimental design

Expression of the three exons 1 present in the majority of GR mRNA in the thymus (1_1, 1_6 and 1_{10}) was studied in the experiments described in this chapter. The thymi used were harvested from the same animals described in Chapters 4 and 5. For details of the adrenalectomy and glucocorticoid replacement see section 2.2.1.

The experimental design was the same as that described for the experiments in Chapter 5, using an RNase protection analysis to provide accurate quantification of GR mRNA levels and *in situ* mRNA hybridisation to provide anatomical detail.

Although autoradiographs are used for illustrative purposes throughout this chapter, analysis of all RNase protection assays was performed using a phosphoimager (section 2.2.11.3).

6.3 Results

6.3.1 Effects of glucocorticoid manipulation on expression of GR mRNA transcripts containing exons 1_1, 1_6 and 1_{10} in the thymus of ST animals

RNase protection analysis using total RNA extracted from thymus showed that short term glucocorticoid replacement significantly lowered the amount of GR mRNA containing exon 1_{10} compared to sham animals (Figure 6.1, Figure 6.2). However, there was no effect of treatment on the proportion of total GR mRNA transcripts containing exon 1_{10} (Figure 6.2).

There was a significant overall effect of treatment on the amount of GR mRNA containing exon 1_1 (Figure 6.3, Figure 6.4), though the individual differences between groups did not reach statistical significance. Since the lanes containing samples from the Sham animals suffered from excessive background (Figure 6.3), the data from these animals was excluded from this analysis. A second analysis was performed comparing the Sham and Adx animals, which showed that adrenalectomy had no effect on exon 1_1 expression (Figure 6.5).

There was no effect of treatment on the proportion of GR mRNA transcripts containing exon 1_1 (Figure 6.4). Similarly, there was no significant effect of treatment on either the amount or the proportion of GR mRNA containing exon 1_6 in the thymi of ST animals (Figure 6.6, Figure 6.7).

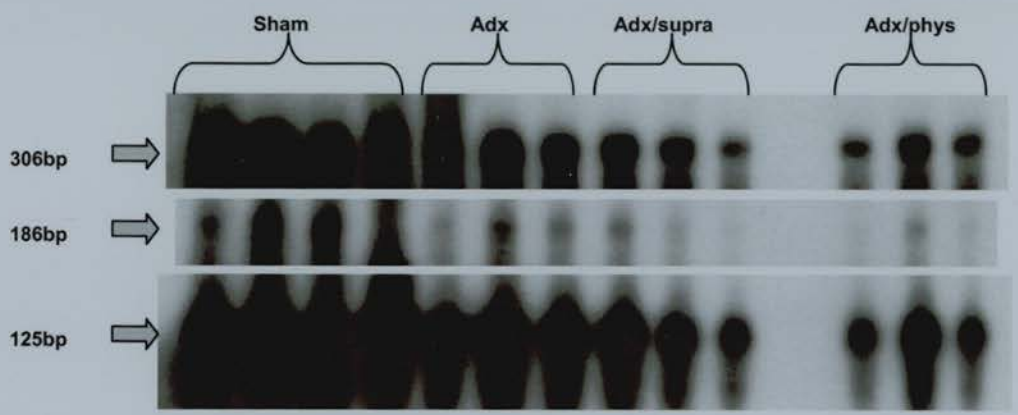


Figure 6.1: representative autoradiograph showing RNase protection analysis of GR mRNA containing exon 1_{10} in thymus of ST animals.

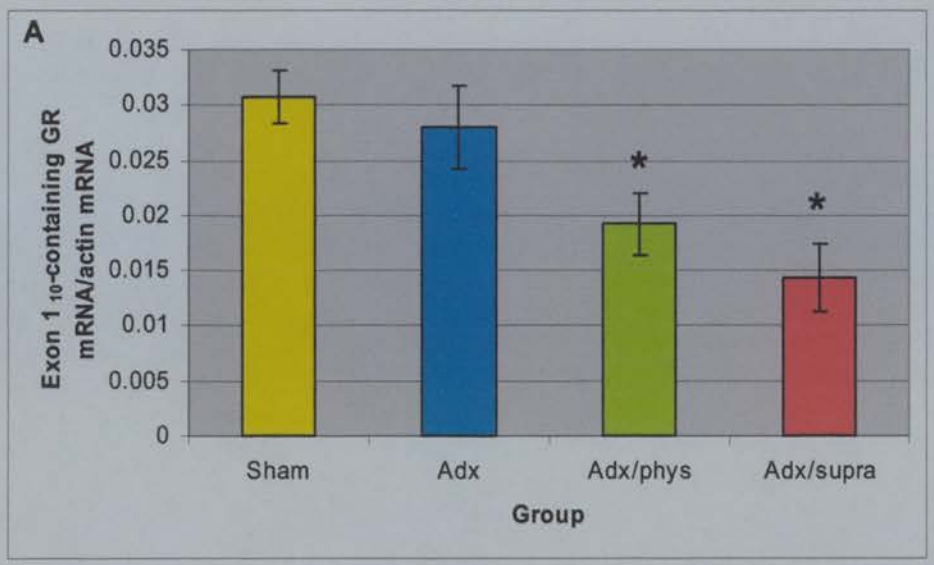
Each lane shows the product of an RPA reaction carried out on 50μg of thymus RNA from a single animal. Arrows indicate the 306bp protected fragment produced by transcripts containing exon 1_{10} and exon 2, the 186bp protected fragment produced by transcripts containing exon 2 but not exon 1_{10} and the 125bp protected fragment produced by actin mRNA. The top panel was taken from a 7d exposure and the centre and bottom panels were taken from an overnight exposure of the same gel.

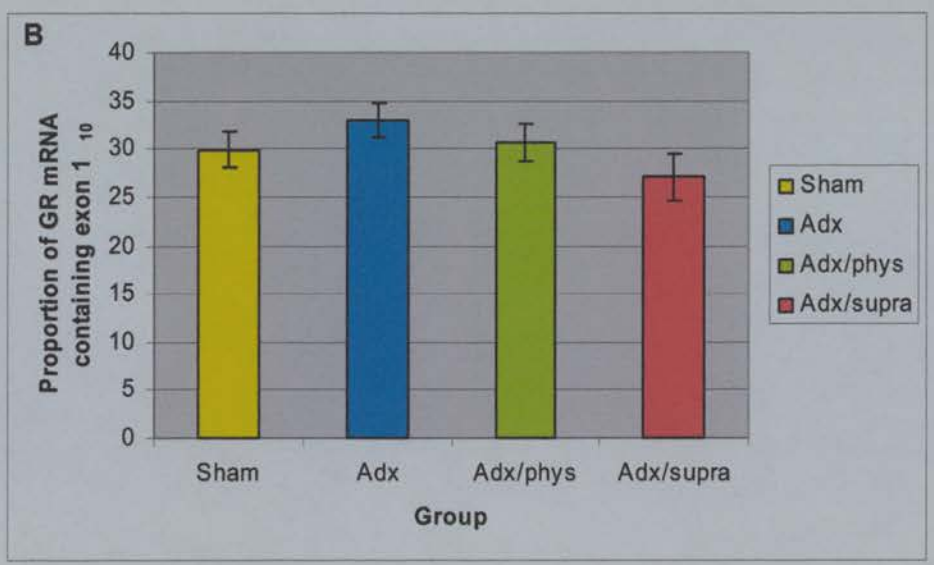
Figure 6.2 (facing page): phosphoimager analysis of RNase protection assay of GR mRNA transcripts containing exon 1_{10} in rat thymus.

Data are presented as mean ± SEM.

A: amount of GR mRNA containing exon 1_{10} in thymus of ST animals expressed as a ratio to actin mRNA. Data were analysed with ANOVA and Tukey's HSD test. There was a significant effect of treatment ($p<0.05$) with lower expression in Adx/phys and Adx/supra animals than in sham-operated controls ($p<0.05$, indicated by *). n=6 per group except Adx/supra n=4 and Sham n=7.

B: proportion of GR mRNA transcripts containing exon 1_{10}. ANOVA showed no significant effect of treatment.


A
Exon 1 10-containing GR mRNA/actin mRNA
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
*
Sham
Adx
Adx/phys
Adx/supra
Group


B
Proportion of GR mRNA containing exon 1 10
40
35
30
25
20
15
10
5
0
Sham
Adx
Adx/phys
Adx/supra
Group
Sham
Adx
Adx/phys
Adx/supra

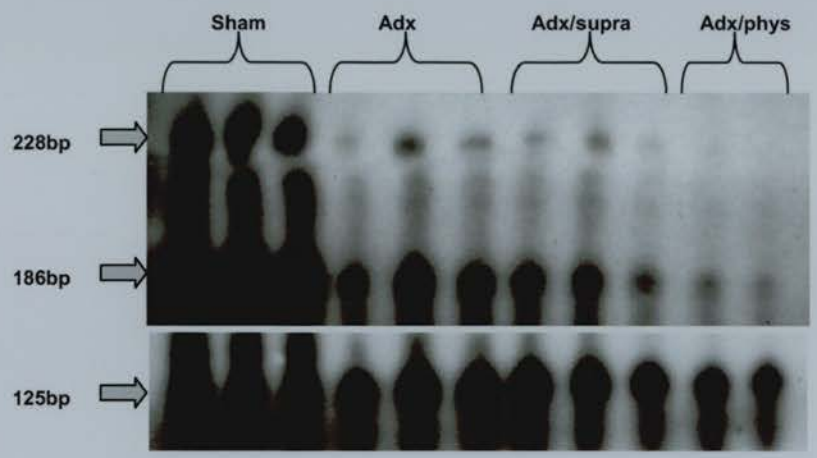


Figure 6.3: representative autoradiograph showing RNase protection analysis of GR mRNA containing exon 1_1 in the thymus of ST animals.

Each lane shows the product of an RPA reaction carried out on 50μg of thymus RNA from a single animal. Arrows indicate the 228bp protected fragment produced by transcripts containing exon 1_1 and exon 2, the 186bp protected fragment produced by transcripts containing exon 2 but not exon 1_1 and the 125bp protected fragment produced by actin mRNA. The upper panel was taken from a 7d exposure while the lower was taken from a 5d exposure.

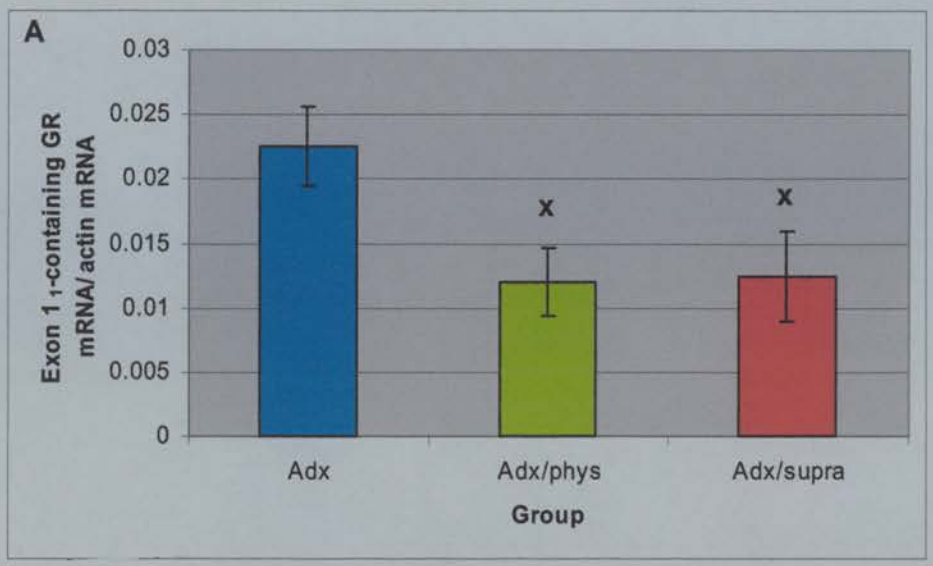
Figure 6.4 (facing page): phosphoimager analysis of RNase protection assay of GR mRNA containing exon 1_1 in thymus of ST animals.

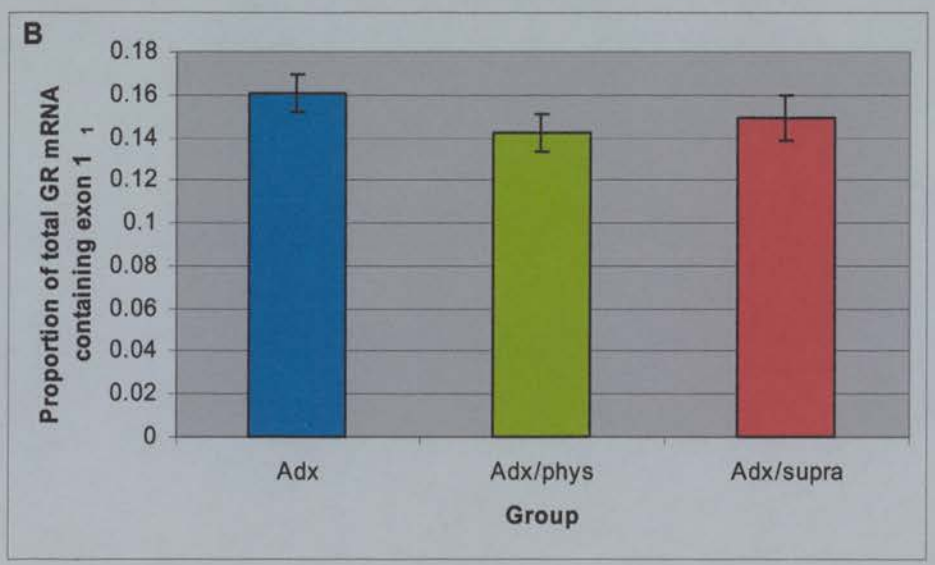
Data are presented as mean ± SEM.

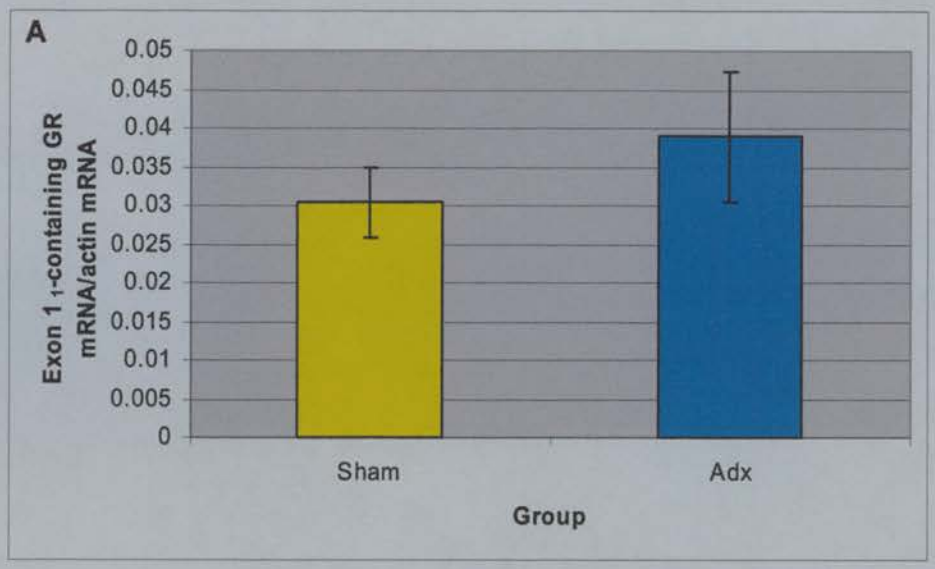
A: amount of GR mRNA containing exon 1_1 expressed as a ratio to actin mRNA. Data were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment ($p<0.05$). Post-hoc analysis using Tukey's HSD Test failed to reveal significant differences between groups, but analysis using Fisher's LSD Test showed that glucocorticoid replacement significantly reduced the amount of GR mRNA containing exon 1_1 compared to adrenalectomy alone ($p<0.05$, indicated by x).

B: proportion of GR mRNA containing exon 1_1.A NOVA showed no significant effect of treatment.

n=5, except Adx/supra where n=4.


A
0.03
0.025
0.02
0.015
0.01
0.005
0
Exon 1 1-containing GR mRNA/ actin mRNA
x
x
Adx
Adx/phys
Adx/supra
Group


B
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Proportion of total GR mRNA containing exon 1 1
Adx
Adx/phys
Adx/supra
Group



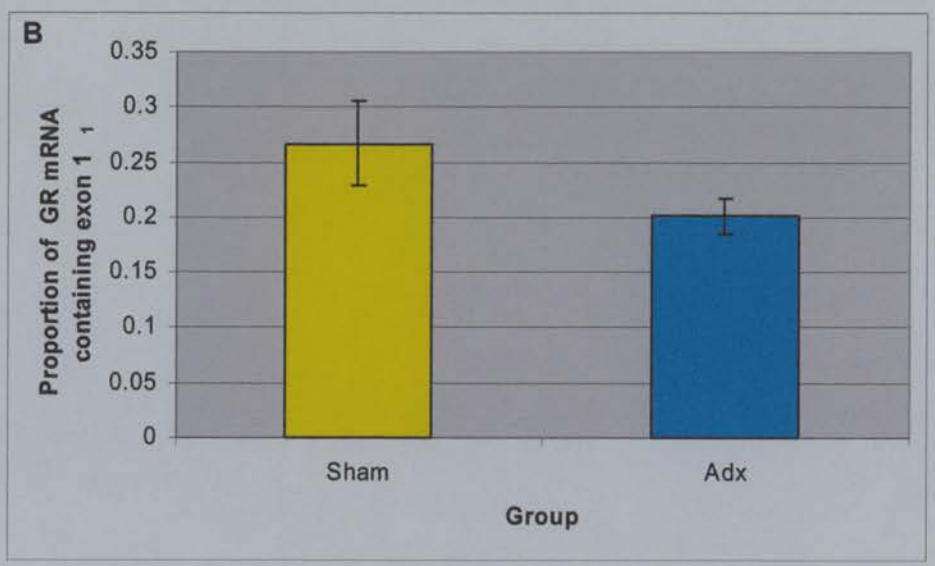


Figure 6.5: phosphoimager analysis of RNase protection assay of GR mRNA containing exon 1_1 in thymus of Adx and Sham ST animals.

A: amount of GR mRNA containing exon 1_1 expressed as a ratio compared to actin mRNA. ANOVA showed no significant effect of treatment.

B: proportion of total GR mRNA containing exon 1_1. ANOVA showed no significant effect of treatment. Adx n=5, Sham n=6.

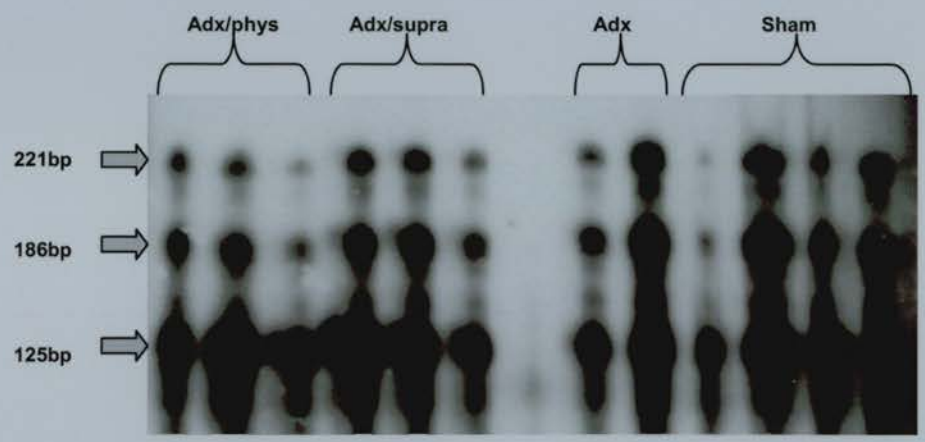
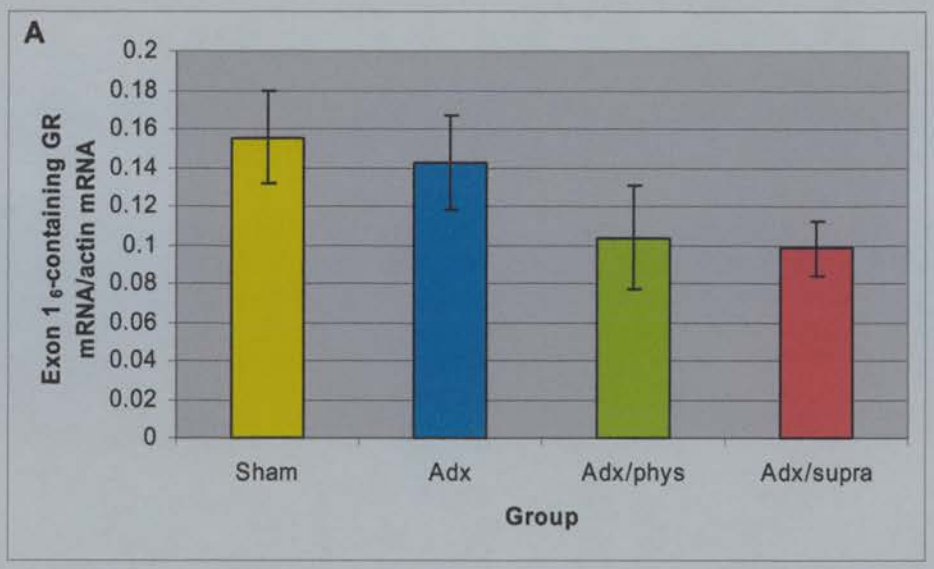


Figure 6.6: representative autoradiograph showing RNase protection analysis of GR mRNA containing exon 1_6 in the thymus of ST animals.

Each lane shows the product of an RPA reaction carried out on 50μg of thymus RNA from a single animal. Arrows indicate the 221bp protected fragment produced by transcripts containing exon 1_6 and exon 2, 186bp protected fragment produced by transcripts containing exon 2 but not exon 1_6 and the 125bp protected fragment produced by actin mRNA transcripts. The autoradiograph was exposed for 5d.



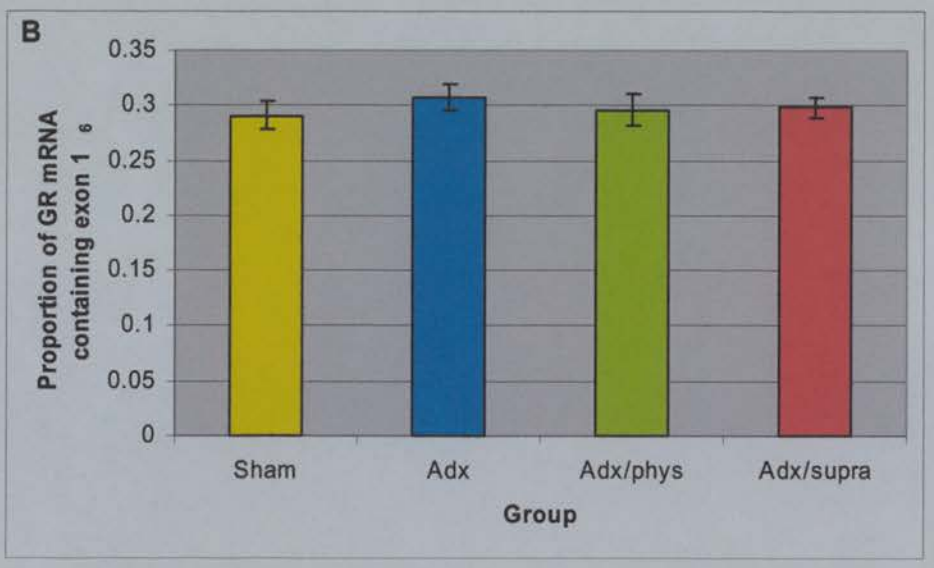


Figure 6.7: phosphoimager analysis of RNase protection assay of GR mRNA containing exon 1_6 in thymus of ST animals.

A: amount of GR mRNA containing exon 1_6 expressed as a ratio to actin mRNA. ANOVA showed no significant effect of treatment.

B: proportion of total GR mRNA containing exon 1_6. ANOVA showed no significant effect of treatment. Adx/phys n=6, Adx/supra and Adx n=5, Sham n=7.

6.3.2 Total GR mRNA levels are significantly affected by glucocorticoid manipulation in the thymus of ST animals

To confirm that short term glucocorticoid manipulations had produced an effect on thymic GR, total GR mRNA levels were assessed in the thymus of the ST animals. Data from the RNase protection analyses for exons 1_1, 1_6 and 1_{10} was combined to determine the abundance of GR mRNA in each experimental group. This showed a significant decrease in total GR mRNA levels in ST animals adrenalectomised and given glucocorticoid replacement compared to sham (Figure 6.8). There was also a strong trend for GR mRNA levels to be significantly lower in the same animals compared to adrenalectomised animals ($p=0.06$).

In situ mRNA hybridization confirmed the distribution of total GR mRNA described in Chapter 3 (Figure 6.9). Unfortunately, initial *in situ* mRNA hybridization experiments revealed a lack of distinct cellular architecture on the emulsion-dipped slides (Figure 6.), with individual thymocytes being all but indistinguishable in the thymic cortex and only occasionally visible in the thymic medulla, although the same protocol had clearly visualized hippocampal and liver tissue architecture (Chapters 4 and 5). However, sufficient detail was visible to partially resolve the tissue architecture and see that silver grains appeared to be evenly distributed over all the cells in both regions (Figure 6.). Grain counting analysis is usually performed by selecting areas for analysis over individual cells. Since these were not clearly visible several relatively large areas of cortex on each section were selected for counting. As the cell density in the medulla varied, it was decided to count over both several cell-dense areas (henceforth referred to as "medulla") and several large areas of medulla similar in size to those selected over the cortex (henceforth referred to as "medulla overall"). The same counting method was used for all the probes used in the thymus.

Analysis of autoradiographs (Figure 6.11) and dipped slides (Figure 6.12) showed a significant decrease in GR mRNA in the cortex and medulla of thymi from ST-adrenalectomised animals given glucocorticoid replacement compared to sham-operated controls. In the ST animals there was no significant effect of adrenalectomy

alone compared to sham-operated controls. In LT animals there was no significant effect of glucocorticoid manipulation on GR mRNA levels or distribution (Figure 6.11F igure 6.12).

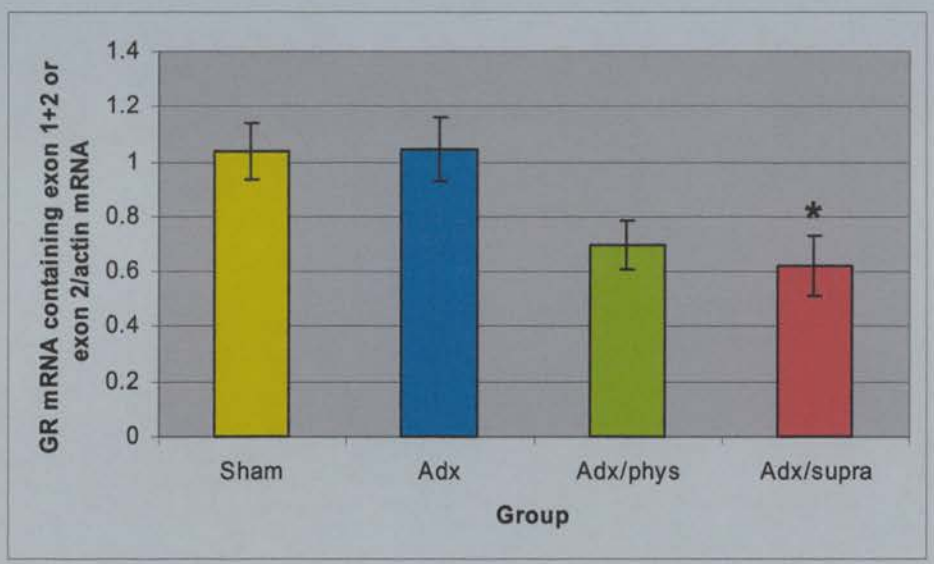


Figure 6.8: phosphoimager analysis of RNase protection assay of total GR mRNA in the thymus of ST animals.

Data (presented as mean ± SEM) is compiled from 3 separate RNase protection analysis experiments using probes specific for exon 2 and one of exons 1_1, 1_6 and 1_{10} of the GR gene. Ratios are calculated as described in section 2.2.11.3. Data from each experiment was normalised to the mean value for the Adx animals, then the values for each animal were averaged across all 4 experiments. Data were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR levels ($p<0.05$), which was significantly lower in the Adx/supra animals compared to Sham ($p<0.05$, indicated by *).

Adx/phys n=6, Adx/supra and Adx n=5, Sham n=7

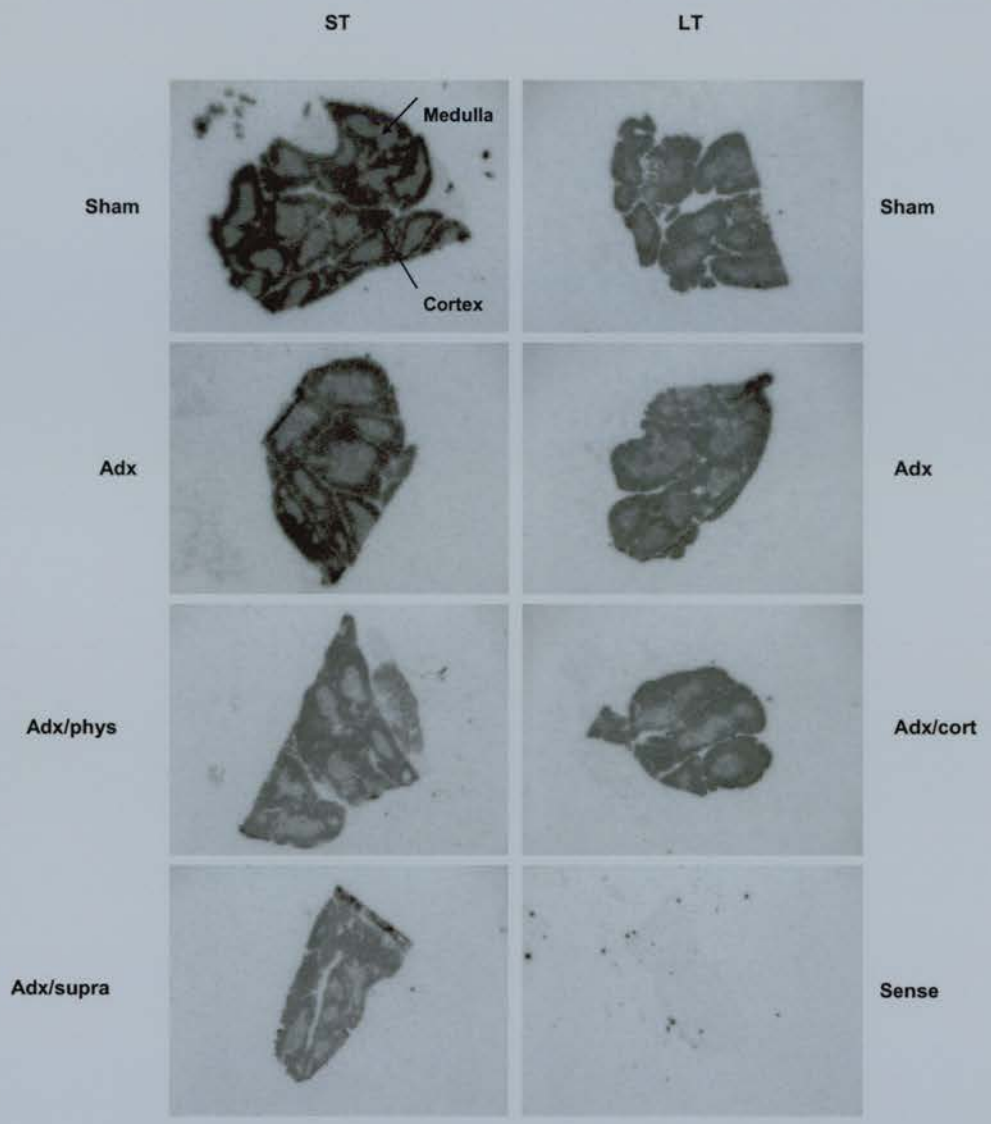


Figure 6.9: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of rat GR cDNA to rat thymus.

ST= short-term adrenalectomised, LT = long-term adrenalectomised, Sham = sham-operated control, Adx = adrenalectomised, Adx/phys =adrenalectomised with physiological glucocorticoid replacement by injection, Adx/supra = adrenalectomised with supraphysiological glucocorticoid replacement by injection, Adx/cort = adrenalectomised with physiological glucocorticoid replacement by subcutaneous pellet, sense = representative sense control.

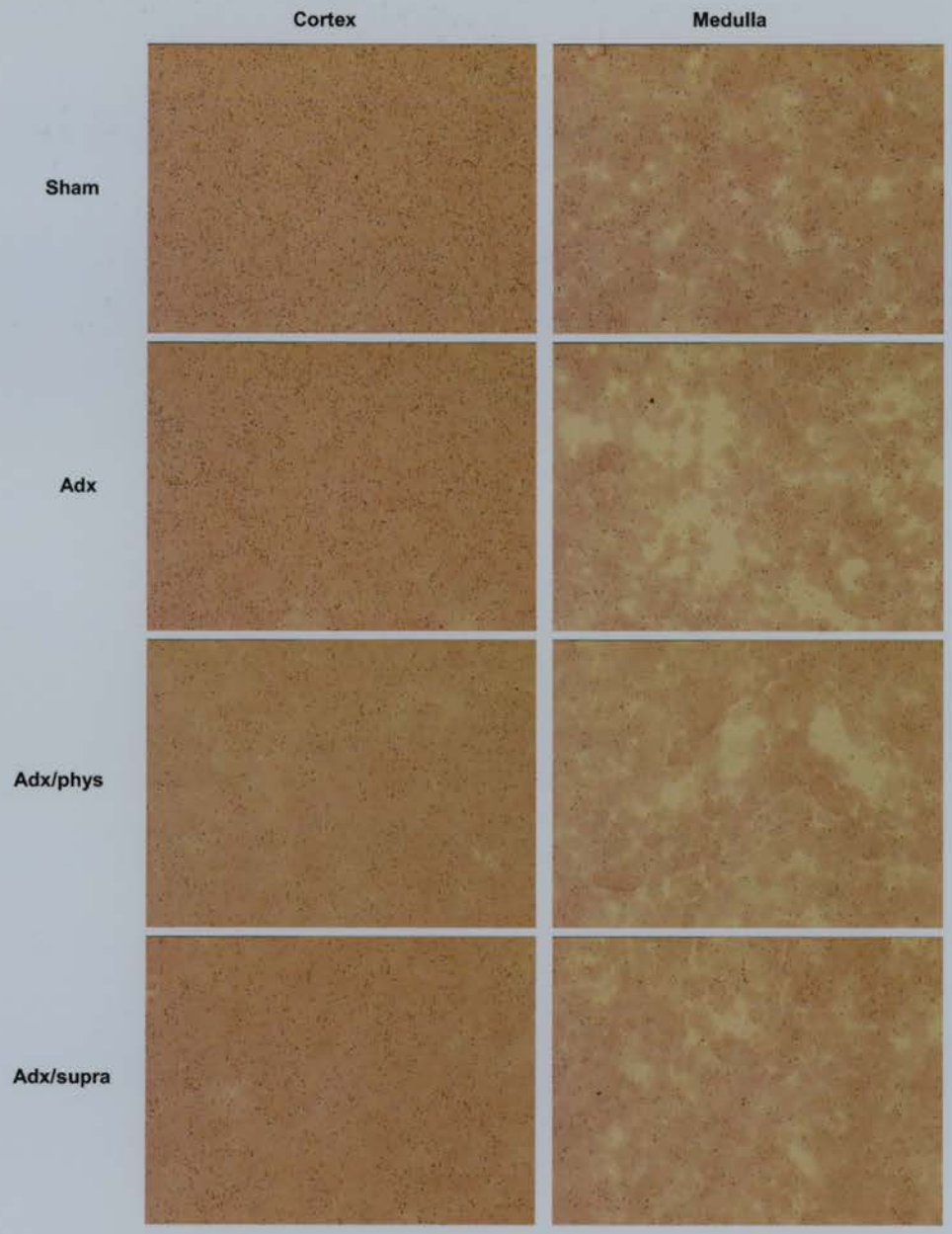


Figure 6.10: representative photomicrographs showing *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of rat GR cDNA to rat thymus.

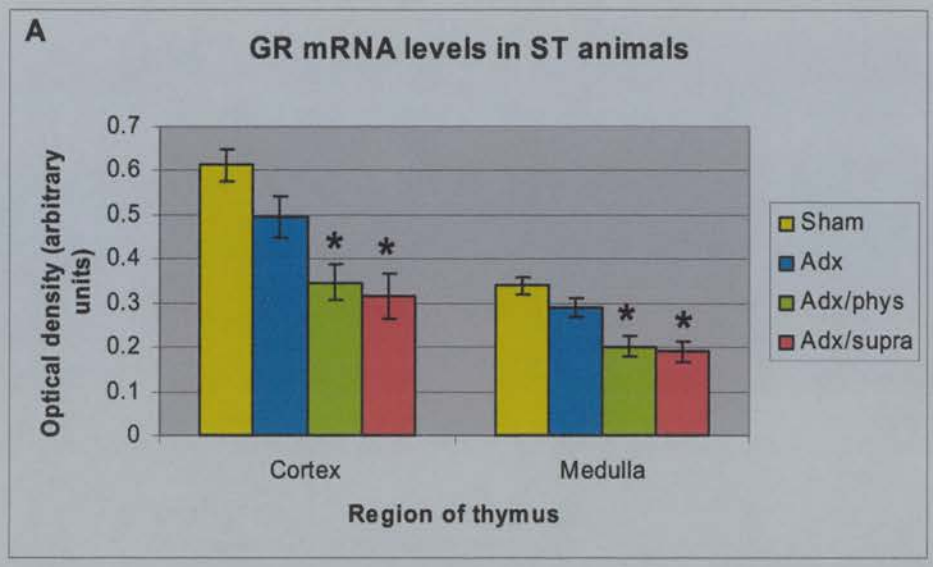
Magnification x 400. Legend as for Figure 6.9.

Figure 6.11 (facing page): densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of rat GR cDNA to rat thymus.

Data are presented as mean ± SEM.

A: ST animals. Data were analysed using ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR expression in cortex and medulla ($p<0.01$), with lower GR mRNA levels in the thymic cortex and medulla of Adx/phys and Adx/supra animals relative to sham-operated controls ($p<0.01$, indicated by *). Also, expression in the Adx/supra animals was lower than that in Adx animals ($p<0.05$, indicated by x). n=6 except Sham where n=8.

B: LT animals. ANOVA showed no significant effect of treatment on GR mRNA levels. n=7, except Adx/cort where n=8.


A
GR mRNA levels in ST animals
Optical density (arbitrary units)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
*
Cortex
Medulla
Region of thymus
Sham
Adx
Adx/phys
Adx/supra

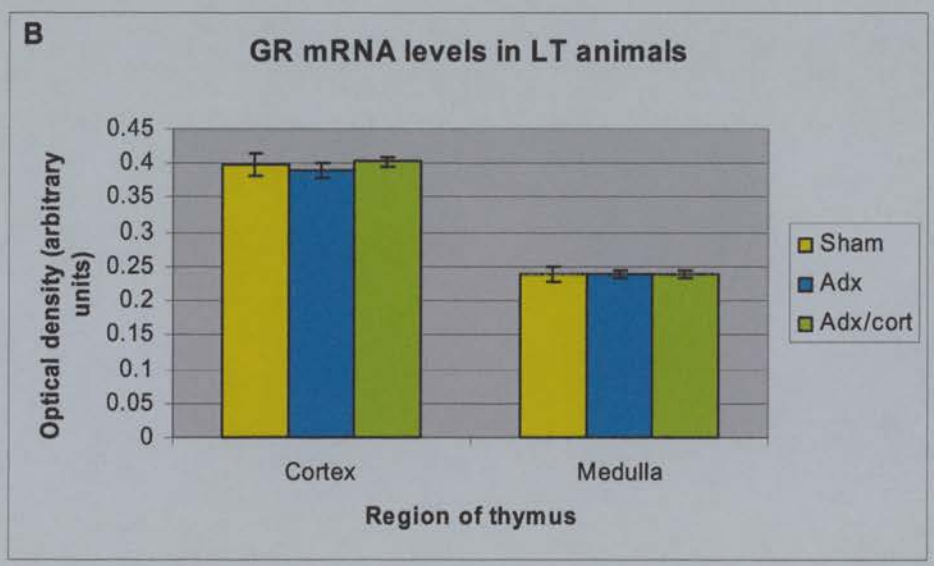
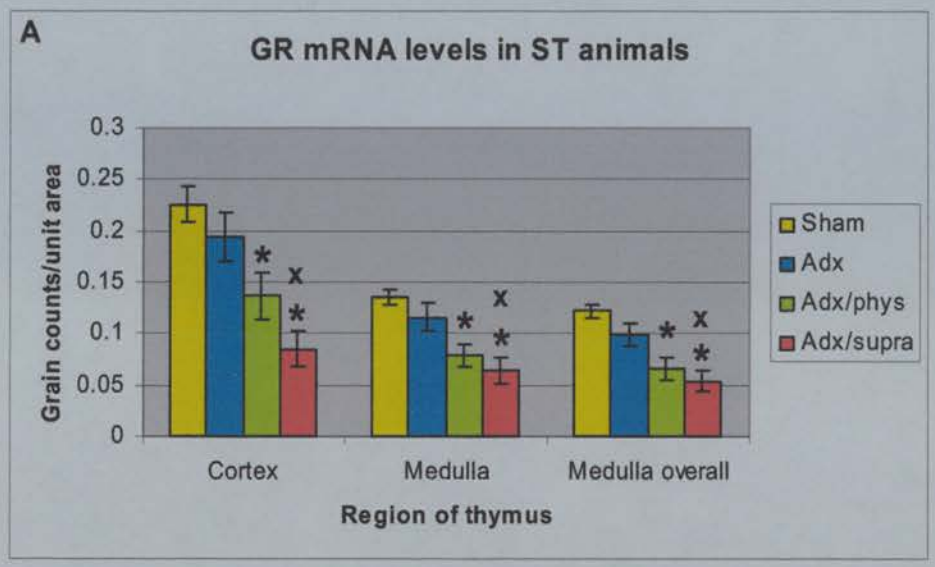

B
GR mRNA levels in LT animals
Optical density (arbitrary units)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Cortex
Medulla
Region of thymus
Sham
Adx
Adx/cort

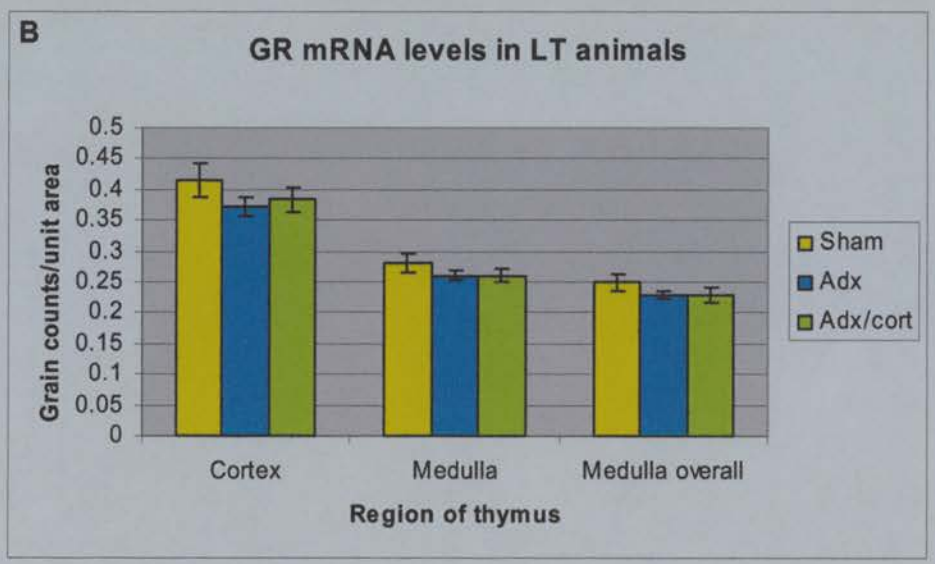
Figure 6.12 (facing page): grain counting analysis of *in situ* mRNA hybridisation of a probe complementary to exons 5-9 of rat GR cDNA to rat thymus.

Data are presented as mean ± SEM.

A: ST animals. Data were analysed by ANOVA and Tukey's HSD test. There was a significant effect of treatment on GR expression in cortex, medulla and medulla overall ($p<0.01$). GR mRNA levels in Adx/phys ($p<0.05$, indicated by *) and Adx/supra ($p<0.01$, indicated by *) animals were significantly lower than in sham operated controls. GR mRNA levels in Adx/supra animals were significantly lower than in Adx animals ($p<0.05$, indicated by x). Adx/phys and Adx n=6, Adx/supra n=5, Sham n=8.

B: LT animals. ANOVA showed no significant effect of treatment on GR mRNA levels. Adx n=6, Adx/cort n=8, Sham n=7.


A
GR mRNA levels in ST animals
Grain counts/unit area
0.3
0.25
0.2
0.15
0.1
0.05
0
Cortex
Medulla
Medulla overall
Region of thymus
Sham
Adx
Adx/phys
Adx/supra


B
GR mRNA levels in LT animals
Grain counts/unit area
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Cortex
Medulla
Medulla overall
Region of thymus
Sham
Adx
Adx/cort

6.3.3 *In situ* mRNA hybridization analysis of the distribution and regulation of GR mRNA transcripts containing variant exons 1 in the thymus

RNase protection analysis and *in situ* mRNA hybridization clearly showed that total GR mRNA levels in the thymus were altered by glucocorticoid manipulations. Furthermore, RNase protection analysis showed that glucocorticoid manipulations altered expression of GR mRNA containing exons 1_{10} and 1_1 in the same way. Since the experiments described in Chapter 3 had shown a regional distribution of GR mRNA containing alternate exons 1 in thymus, it was necessary to determine whether region-specific changes in the expression of GR mRNA transcripts were being diluted in the RNase protection analysis and missed. Thus, *in situ* mRNA hybridization was used to determine whether regulation of expression of these transcripts by glucocorticoids was region-specific.

Analysis of autoradiographs (Figure 6.13, Figure 6.14) showed no significant effect of changes in glucocorticoid levels on expression of GR mRNA transcripts containing exon 1_1 in ST or LT animals. Grain counting analysis gave similar results (data not shown).

Also, analysis of autoradiographs (Figure 6.15F igure 6.16) showed no significant effect of glucocorticoid manipulation on expression of GR mRNA containing exon 1_{10} in the thymic cortex or medulla of ST or LT animals. Grain counting analysis gave similar results (data not shown).

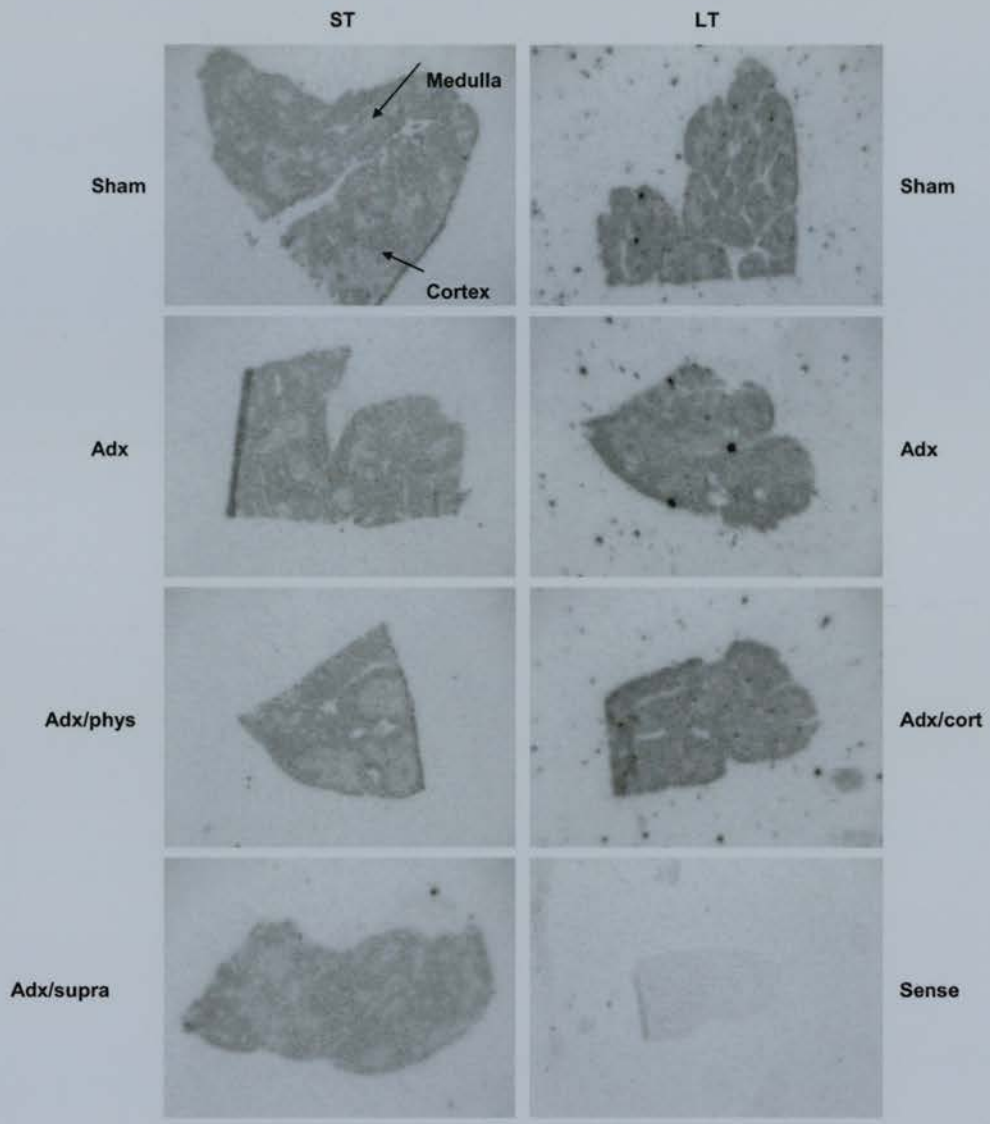


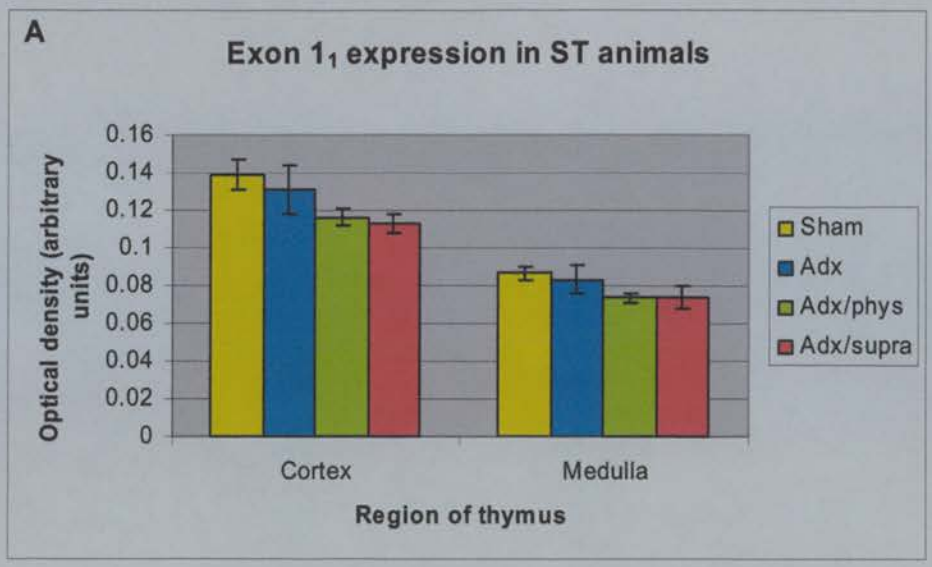
Figure 6.13: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_1 of the GR gene in rat thymus. Legend as for Figure 6.9.

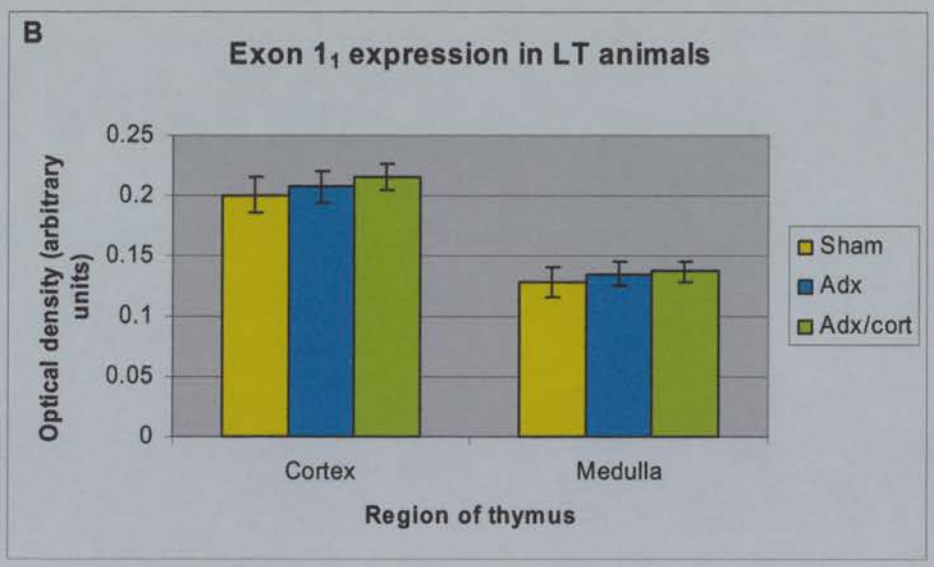
Figure 6.14 (facing page): densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_1 of the GR gene in rat thymus.

Data are presented as mean ± SEM.

A: ST animals. ANOVA showed no significant effect of treatment on expression. n=6, except Sham where n=8.

B: LT animals. ANOVA showed no significant effect of treatment on expression. Adx n=6, Adx/cort n=8, sham n=7.


A
Exon 1$_1$ expression in ST animals
Optical density (arbitrary units)
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Cortex
Medulla
Region of thymus
Sham
Adx
Adx/phys
Adx/supra


B
Exon 1$_1$ expression in LT animals
Optical density (arbitrary units)
0.25
0.2
0.15
0.1
0.05
0
Cortex
Medulla
Region of thymus
Sham
Adx
Adx/cort

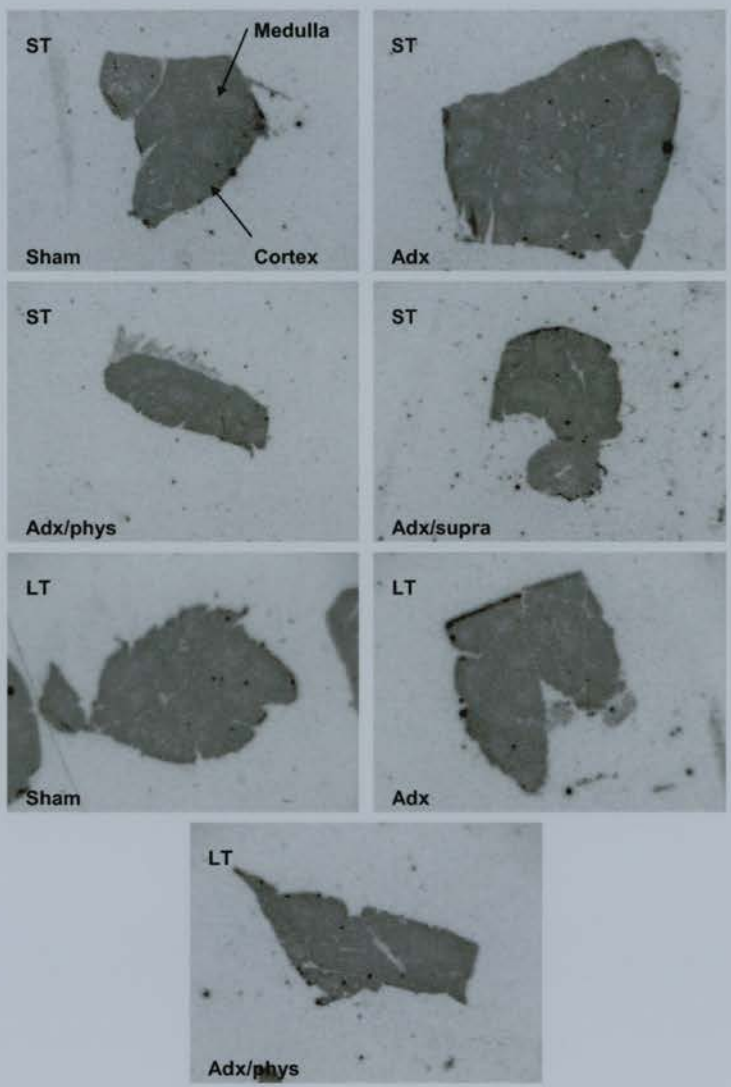


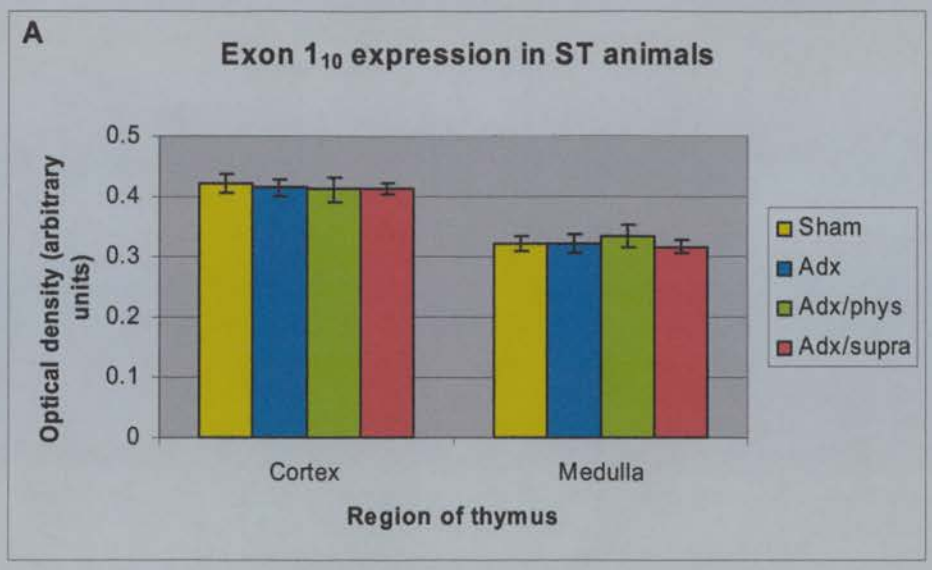
Figure 6.15: representative autoradiographs showing *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to the thymus of ST and LT animals. Legend as for Figure 6.9.

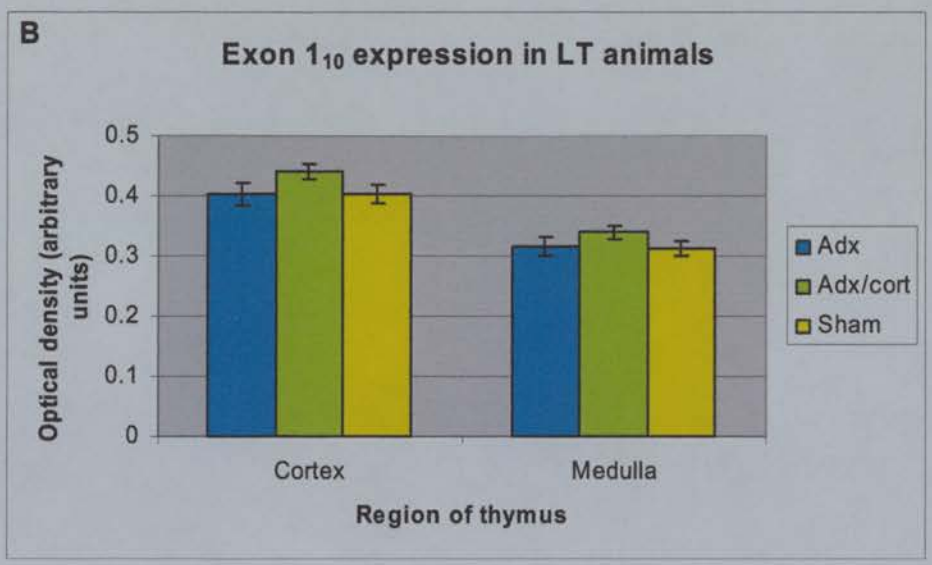
Figure 6.16 (facing page): densitometric analysis of *in situ* mRNA hybridisation of a probe complementary to exon 1_{10} of the GR gene to rat thymus.

Data are expressed as mean ± SEM.

A: ST animals. ANOVA showed no significant effect of treatment on expression. n=6, except Sham where n=7.

B: LT animals. ANOVA showed no significant effect of treatment on expression. n=8, except Adx where n=7.


A
Exon 1$_{10}$ expression in ST animals
Optical density (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0
Cortex
Medulla
Region of thymus
Sham
Adx
Adx/phys
Adx/supra


B
Exon 1$_{10}$ expression in LT animals
Optical density (arbitrary units)
0.5
0.4
0.3
0.2
0.1
0
Cortex
Medulla
Region of thymus
Adx
Adx/cort
Sham

6.3.4 LT adrenalectomised animals receiving glucocorticoid replacement show reduced thymus weight.

There was a significant reduction in thymus weight in LT Adx animals given corticosterone replacement compared to those adrenalectomised alone (Figure 6.17). However, there was no effect of adrenalectomy on thymus weight compared to sham-operated controls (Figure 6.17).

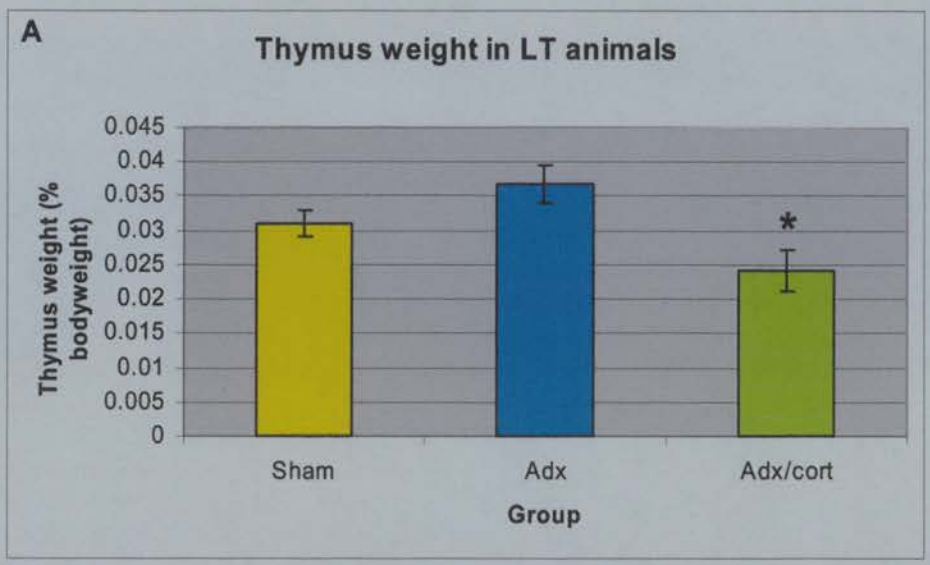
There was no effect of glucocorticoid manipulation on thymus weight in ST animals (Figure 6.17). However, in ST sham animals thymus weight formed an approximately 4-fold greater percentage of the animal's bodyweight than in LT sham animals (Figure 6.17). The most likely explanation for this is that when the ST tissues were harvested, variable amounts of fat were dissected out along with the thymus due to unfamiliarity with the exact anatomy of the area, rendering the data unreliable.

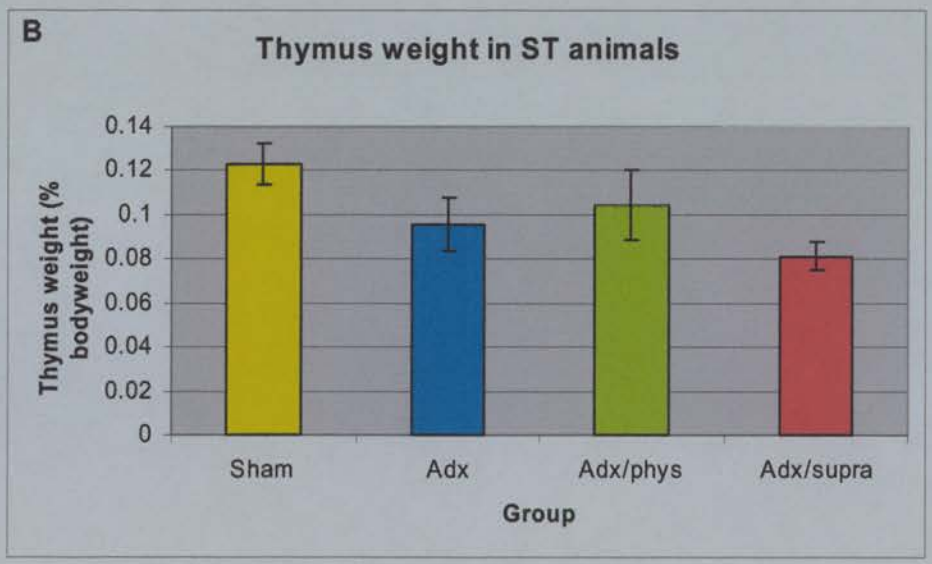
Figure 6.17 (facing page): thymus weights of LT and ST animals expressed as a percentage of bodyweight at the time of tissue harvesting.

Data (presented as mean ± SEM) were analysed by ANOVA and Tukey's HSD test.

A: LT animals. There was a significant effect of treatment on thymus weight ($p<0.05$). Thymus weights were significantly lower in Adx/cort animals compared to Adx animals ($p<0.01$, indicated by *). Adx n=6, Adx/cort n=8, sham n=7.

B: ST animals. ANOVA showed no significant effect of treatment on thymus weight. n=6 except sham n=7.


A
Thymus weight in LT animals
Thymus weight (% bodyweight)
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
*
Sham
Adx
Adx/cort
Group


B
Thymus weight in ST animals
Thymus weight (% bodyweight)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
Sham
Adx
Adx/phys
Adx/supra
Group

6.4 Discussion

Supraphysiological or physiological glucocorticoid replacement in ST adrenalectomised animals decreased the level of GR mRNA in thymus containing exons 1_1 or 1_{10} compared to sham-operated controls. Also, there was a strong trend for the level of GR mRNA containing exon 1_6 to be reduced by the same glucocorticoid manipulations. In each case, the magnitude of the effect is similar to the magnitude of the effect on total GR mRNA. The effects of glucocorticoid manipulations on total GR mRNA expression are in agreement with the results of previous studies (Peiffer et al., 1994; Spencer et al., 1991). Thus one can rule out a dramatic and differential downregulation of GR mRNA containing exon 1_1, 1_6 or 1_{10} by glucocorticoids.

GR mRNAs containing the 3 variant exons 1 studied here made up a total of 78-90% of the total population of GR mRNAs, indicating that transcripts containing other exons 1 are likely to be expressed in thymus. If so, these exons 1 must be novel, since none of the other known exons 1 were detected in thymus by RNase protection analysis (McCormick et al., 2000). However, there was no effect of treatment on the percentage of the total population of GR mRNAs containing each of the exons 1 studied, suggesting that an increase or decrease in the expression of a novel exon 1 cannot account for the change in total GR mRNA seen with adrenalectomy and glucocorticoid replacement, unless it is regulated in exactly the same way as the known exons 1.

Interestingly, these experiments do not show a glucocorticoid-mediated increase in expression of total GR or GR mRNA containing exon 1_1, which might have been expected since glucocorticoids have been shown to increase expression of total GR mRNA (Antakly et al., 1989; Antakly et al., 1990; Ashraf et al., 1991; Denton et al., 1993; Eisen et al., 1988) and GR mRNAs containing exon 1A (Breslin et al., 2001) in human CEM-C7 T cells. This raises the question of why glucocorticoids seem to

have opposite effects on total GR and exon 1A expression in this cell line and the intact thymus.

One possible explanation may be that glucocorticoid upregulation of GR mRNA containing exon 1A and total GR mRNA is confined to a subset of T cells. Indeed, there may be a different role for exon 1_1 (1A) and its associated promoter in different cell types (Breslin et al., 2001). Regulation of GR levels by changes in the activity of promoter 1A has been implicated in glucocorticoid-induced T cell apoptosis and T cell maturation (Chen et al., 1999b; Chen et al., 1999a). Also, exon 1A has been suggested to encode membrane-associated GR (mGR) or direct GR to the plasma membrane (Chen et al., 1999b), although it is unclear how this might occur since the in-frame stop codon at the start of exon 2 means that transcripts containing different exons 1 will produce identical GR proteins. However, preliminary work from our laboratory has suggested that GR mRNA containing exons 1_1 and 1_{10} are expressed at similar levels (≈20-25% and 50-70% of total GR mRNA respectively) in whole thymus, thymocytes, thymic epithelium and mature splenic T cells (Dammermann, A., unpublished observations). Additionally, both mRNA variants showed the same distribution as total GR mRNA in the *in situ* mRNA hybridisation experiments described here, even when subject to glucocorticoid manipulations. At a gross level, both variants showed even expression throughout the thymic cortex and medulla. However, the *in situ* mRNA hybridisation experiments performed here do not allow the use of double staining with markers for thymic cell types. Further work using digoxygenin-labelled probes coupled with immunohistochemistry could allow this and resolve the question of whether the variant exons 1, particularly 1_1, respond differently to glucocorticoid manipulations in different cell types. Also, sorting of thymocytes into different cell populations ($CD4^+CD8^+$, $CD4^-CD8^-$, $CD4^+CD8^-$, $CD4^-CD8^+$) and separation from cells of the thymic epithelium ($CD45^-$) could allow investigation of whether expression of GR mRNAs containing variant exons 1 varies developmentally and whether glucocorticoid manipulations affect their expression differently at different stages of T cell maturation.

Alternatively, there may have been loss of a subset of thymocytes that upregulate GR in response to glucocorticoid administration. Glucocorticoids induce apoptosis in

thymocytes and T cell lines (reviewed in section 1.9.5)). Also, glucocorticoid-induced upregulation of GR may occur during thymocyte apoptosis, since induction of GR expression in glucocorticoid-resistant T cells confers glucocorticoid sensitivity (Ramdas et al., 1999) and microarray analysis of gene expression during T cell apoptosis found that increased GR expression is a primary phenomenon in apoptosis induced by both short and long-term glucocorticoid administration (Tonko et al., 2001). Thus it is possible that glucocorticoid administration induced apoptosis in glucocorticoid-sensitive cells that might upregulate GR in response to glucocorticoids, leaving cells which may be resistant to apoptosis because they downregulate GR levels. This hypothesis is supported by the reduction in thymus weight in the LT adrenalectomised animals receiving glucocorticoid replacement compared to those adrenalectomised alone, which may indicate thymic involution in response to glucocorticoid administration.

Another possible explanation for why the cultured cells may respond differently to glucocorticoids is that they are away from the complex hormonal microenvironment of the thymus (reviewed in section 1.9.5.2)). Also, since the cell lines are transformed (i.e. tumour) cells they may respond differently to glucocorticoids, although down-regulation of GR (Denton et al., 1993) and exon 1A (Breslin et al., 2001) by glucocorticoids has been shown in a B cell line, which makes this a less likely explanation.

While expression of GR mRNA transcripts containing exons 1_1 and 1_6 in the thymus of Sham animals was comparable to that described previously for control rats, transcripts containing exon 1_{10} made up a lower proportion of total GR mRNA than reported previously (McCormick et al., 2000). As considerable variation exists in the proportion of transcripts containing exon 1_{10}, with work from our laboratory suggesting that they form between 53% (McCormick et al., 2000)and 80% (Dammermann, A., unpublished observations) of the total GR mRNA population, this difference may be due to experimental variability.

There were differences between the results of RNase protection analysis and *in situ* mRNA hybridisation analysis of the effect of glucocorticoid manipulation on

regulation of exons 1_1 or 1_{10}, with the former showing a significant effect and the latter not. There are several possible reasons for this discrepancy. RNase protection analysis is highly quantitative, whereas in situ mRNA hybridisation is semiquantitative at best. As discussed in section 6.2 there were technical problems with the integrity of the tissue for the *in situ* mRNA hybridisation experiments, which may have affected the results. Nevertheless, the *in situ* mRNA hybridisation does rule out a dramatic regional difference in the effects of glucocorticoids on expression of total GR mRNA or GR mRNA containing these variant exons 1.

In contrast to the effects after 72h, 3 weeks of physiological glucocorticoid replacement had no effect on thymic GR levels. The most likely explanation for this is that the LT animals had adapted to the glucocorticoid manipulation, leading to the level of GR in the thymocytes returning to normal. 2 previous studies have reported that the effects of adrenalectomy on hippocampal GR are abolished 2 weeks post-adrenalectomy (Holmes et al., 1995b; Reul et al., 1989). Also, rats which failed to suppress their plasma corticosterone levels in response to dexamethasone after acute stress showed normal suppression after 4 weeks of daily exposure to the same stressor (Mizoguchi et al., 2001). These data suggest that adaptation to changes in circulating glucocorticoid levels is possible. Further work using intermediate time points would help to resolve this issue.

7. Discussion

The glucocorticoid receptor is highly expressed in the hippocampus, liver and thymus, where it has an important physiological role. Glucocorticoid regulation of GR levels is well-recognised in all these tissues, both associated with perinatal programming (section 1.7.3) and dynamic regulation of GR levels in the adult animal (section 1.7.2.1). The aims of this thesis were to further characterise the expression of variant exons 1 of the GR gene in these glucocorticoid target tissues and investigate whether changes in alternate promoter usage are associated with glucocorticoid autoregulation of GR levels.

Variant mRNAs can arise from a single gene by 4 basic mechanisms: initiation of several primary transcripts at alternative promoters, differential termination or 3' post-translational processing, alternative splicing of the same primary transcript or recombination of genomic sequences ((Ayoubi and VanDeVen, 1996) and section 1.10.2). Of these, the use of alternative promoters is the most likely to lead to the formation of mRNAs with variant 5' regions of the type seen with the GR gene. Tissue-specific alternate promoter usage leading to the production of mRNAs with multiple untranslated 5' regions is seen for many genes including the human oestrogen receptor gene (Flouriot et al., 1998) and the mineralocorticoid receptor gene (Zennaro et al., 1995). Also, alternate exons 1 of the GR gene are associated with promoter activity in transfected cells (Breslin et al., 2001; McCormick et al., 2000), again suggesting that GR exon 1 variants arise due to alternative promoter usage.

The experiments described in chapter 3 demonstrated tissue and region-specific differences in relative expression of variant exons 1 of the mouse and rat GR genes, consistent with the results of previous studies (McCormick et al., 2000; Strahle et al., 1992). Tissue-specific expression of GR variant mRNAs may be due to changes in promoter usage in different tissues, with some (e.g. the promoter associated with the rat exon 1_7) proposed to be tissue-specific while others (e.g. the promoters associated

with exons 1_{10} and 1_6) proposed to be constitutively active (McCormick et al., 2000). Similar alternate promoter usage is found in other nuclear hormone receptor genes such as the rat MR gene (Kwak et al., 1993) and human and mouse ER genes (Kos et al., 2000; Osterlund et al., 2000).

However, it is debatable whether the expression of the majority of exons 1 is truly tissue-specific, since GR mRNA containing all of the exons 1 except exons 1_1 and 1_4 was detected in all the rat and mouse tissues studied. Most of the other exons 1 in the rat are within the 3kb CpG island that lies between approximately -1620 and -4520 relative to the translation start at +1 in exon 2 (McCormick et al., 2000). It is possible that transcription starts at multiple sites throughout the CpG island, with the specific mRNA produced depending on the first available splice donor site encountered by the transcriptional machinery. This hypothesis is supported by the observation that several of the exons 1 in the CpG island exhibit multiple transcription start sites (McCormick et al., 2000). Such a mechanism might lead to expression of all the variant mRNAs associated within the CpG island, with relative levels of promoter usage determined by the tissue-specific mix of transcription factors present. Since a substantial proportion of the GR mRNA in immune tissues (≈25%) contains exon 1_1 and this exon lies a considerable distance 5' of the other exons 1 in rat (McCormick et al., 2000), mouse (Strahle et al., 1992) and human cells (Breslin et al., 2001) it is plausible that the promoter associated with it might be regulated differently those associated with the other exons 1, perhaps by a tissue-specific transcription factor. However, exon 1_4 is much less abundant and is located much closer to the other exons 1 on the genomic DNA at the 5' end of the CpG island (McCormick et al., 2000). This raises the question of how the promoter associated with exon 1_4 might be regulated differently to the other CpG island promoters. Interestingly, when the sequence of the GR gene 5' flanking region is examined using GRAIL (Uberbacher and Mural, 1991), the area around exon 1_4 is less strongly predicted to be a CpG island than that around the more 3' exons 1. This raises the possibility that there is a difference in methylation status of the DNA in these two areas. The 5' area around exon 1_4 might be less protected from methylation during development by binding of transcription factors (perhaps including GR) than the more 3' area, which could

account for the lack of exon 1_4 expression in some tissues. Apart from exon 1_4, it appears that there is transcription of all the other exons 1 in the CpG island.

The observed tissue-specific differences in the relative expression of variant GR mRNAs raises the question of what their physiological relevance might be. The existence of alternate promoters can confer greater flexibility in the regulation of genes (section 1.10.2). Indeed, changes in the activity of specific GR promoters in liver and hippocampus has been suggested as a mechanism for perinatal programming of GR expression by glucocorticoids (McCormick et al., 2000). This led to the hypothesis that similar changes would be seen during glucocorticoid autoregulation of GR levels in adult animals. However, in the studies described here GR mRNAs containing variant exons 1 were either regulated by glucocorticoids in the same manner as total GR mRNA or were unresponsive to glucocorticoid manipulations. Thus although expression of all variant GR exons 1 appears to be regulated by glucocorticoids, the changes in total GR mRNA expression seen with glucocorticoid manipulations cannot be accounted for by changes in expression of a specific exon 1. This, in turn, suggests that the mechanism by which glucocorticoids regulate GR expression may be by uniformly altering activity of all the promoters of the GR gene, rather than by effecting changes in the activity of specific alternate promoters in different tissues. This is perhaps surprising since alternate promoters of the MR gene are reported to be specifically regulated by adrenocorticosteroids, with the expression of rat αMR mRNA specifically upregulated in hippocampus by adrenalectomy (Kwak et al., 1993) and the P2 promoter of the human MR gene being stimulated by aldosterone in a dose-dependent manner in transfection experiments (Zennaro et al., 1996). There is little information available regarding the location of glucocorticoid-responsive sequences in the GR gene promoter region, which might indicate whether GR is likely to regulate transcription at all or a subset of the promoters and provide insights into the mechanisms of GR regulation. Leclerc *et al* identified a 500bp glucocorticoid responsive region upstream of exon 1C of the human GR gene, which they report is responsible for dexamethasone-mediated downregulation of GR levels in cultured cells (Leclerc et al., 1991). However, no footprint analysis was performed and sequence analysis of the region failed to locate

potential GR binding sites (Leclerc et al., 1991). If footprinting analysis were to demonstrate the presence of GR binding sites throughout the promoter region, this might support the hypothesis that glucocorticoid autoregulation affects the activity of all promoters equally. The data from the experiments described in this thesis suggest that changes in activity of specific promoters may be involved in determining the tissue-specific "set-point" of GR expression and in perinatal programming, while glucocorticoid autoregulation of GR levels involves changes in the activity of all the alternate promoters. Further experiments are required to test this hypothesis.

The mechanism by which perinatal programming might induce changes in alternate promoter usage is unclear. Experiments to further investigate the effects of perinatal glucocorticoid manipulations could address this issue, perhaps by investigating the methylation status or chromatin structure of the DNA associated with the various promoters, either of which might be modified in programmed animals. Interestingly, it has recently been proposed that increased levels of NGFI-A and AP-2 in hippocampal neurones after neonatal handling might lead to reduced methylation of the GR gene promoter region and permanently increased GR gene expression (Weaver et al., 2001), although this is highly speculative. The methylation status of the promoter regions could be assessed in a variety of ways e.g. by methylation-specific PCR (Herman et al., 1996). Preliminary data on the chromatin structure of the GR gene in rat liver suggests that a region of DNase I sensitivity lies close to or within exon 1_{10}. This would be consistent with the chromatin being open in this transcriptionally active area and might suggest that modifications of chromatin structure influence variant exon 1 expression. Work is currently underway in our laboratory to further characterise the chromatin structure of the GR promoter region in liver cells and a variety of cell lines.

Some of the variant GR mRNAs that were identified by RT-PCR could not be detected by RNase protection analysis in the same tissue, suggesting that these exons are probably either expressed in a small proportion (1-2%) of the GR mRNAs in all cells in the tissue or are present at higher levels in a subpopulation of cells. This question could be addressed by investigating the expression of variant GR mRNAs in different cells, for example by sorting subpopulations of thymocytes or hepatocytes

or by performing single-cell RT-PCR. If the transcripts are present in all cells, it is likely that glucocorticoid-induced variations in usage of the promoters associated with the more common transcripts would be more physiologically relevant than would variations in those associated with the rarer transcripts. However, levels of GR mRNA may not be directly proportional to levels of GR protein in a given tissue since some post-translational control of GR levels may occur (Dong et al., 1988; Rosewicz et al., 1988). This raises the intriguing possibility that the pattern of alternate variant exon 1 expression in a tissue could control the level of GR protein in the cell by generating a tissue-specific mix of GR mRNAs which might have different stabilities or translation efficiencies. The mRNA stability of several genes is regulated during development (e.g. the c-myc proto-oncogene), in response to hormonal cues (e.g. various serum protein mRNAs) and various environmental factors like hypoxia, hypocalcaemia and tissue injury (reviewed in (Guhaniyogi and Brewer, 2001)). Acute stress has been reported to cause a reduction in hippocampal GR mRNA levels without affecting levels of GR heteronuclear RNA (Paskitti et al., 2000) and treatment of rat hepatoma cells with the cAMP analogue 8-bromo-cAMP causes a 2.5-fold increase in GR mRNA stability, suggesting that changes in GR mRNA levels may be mediated by changes in mRNA stability (Dong et al., 1989). Also, glucocorticoids have been shown to decrease the stability of mRNAs encoding the type I procollagen (Raghow et al., 1986) and cyclin D3 (Reisman and Thompson, 1995) genes. mRNA stability may be affected by several determinants in the 3' untranslated, coding and 5' untranslated regions of the mRNA (reviewed in (Guhaniyogi and Brewer, 2001)). Since the 5' UTR of mRNAs can alter stability by either containing translation-inhibiting stem loops or by containing binding sites for RNA-stabilising proteins (reviewed in (Guhaniyogi and Brewer, 2001)) and alternate exon 1 expression alters the 5' end of the GR mRNA (McCormick et al., 2000), glucocorticoid-induced changes in GR mRNA stability could be a plausible mechanism for autoregulation of GR levels. A fruitful line of enquiry could be to assess the stability of variant GR mRNAs e.g. by actinomycin or *in vitro* decay assays.

The existence of alternate exons 1 might also be relevant to the control of the translation, stability or activity of GR. Dexamethasone reduced the half-life of GR protein in rat hepatoma cells (Dong et al., 1988); one possible explanation for this could be that dexamethasone administration is inducing the expression of a GR protein variant with lower stability. Alternatively, dissociation from HSP90 might leave GR more susceptible to proteosome-mediated degradation (Wallace and Cidlowski, 2001). The recent discovery of human GR isoforms with different c-terminal ends (Yudt and Cidlowski, 2001) and the existence of the GRβ isoform in human cells (Encio and Detera-Wadleigh, 1991; Hollenberg et al., 1985; Oakley et al., 1996) raises the possibility of the existence of GR protein isoforms with different stability, although the physiological relevance of the GRα/β and A/B isoforms remains to be established.

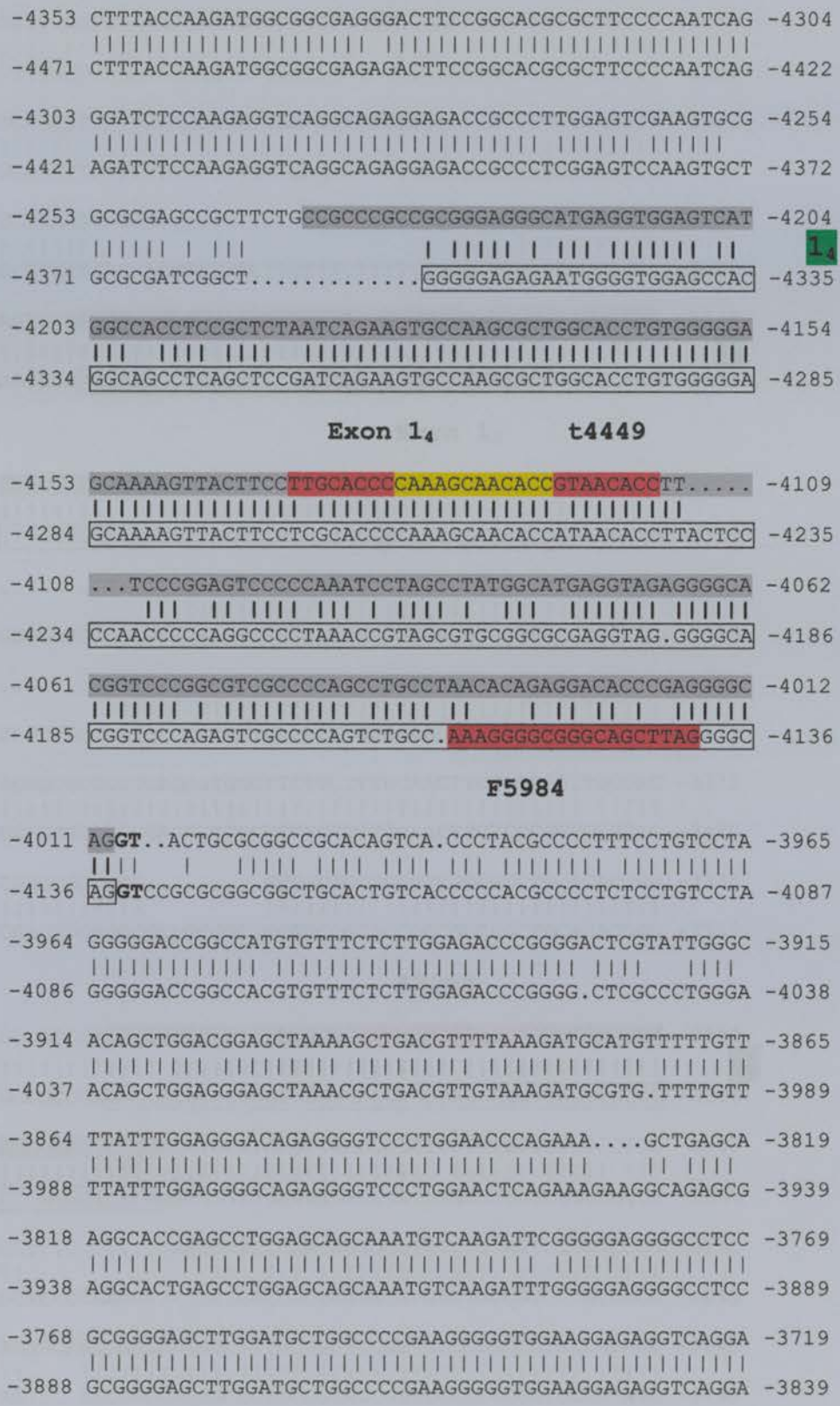
In summary, the experiments described in this thesis have shown that the structure of the GR gene is highly conserved between rats and mice. Furthermore, most variant exons 1 of the mouse and rat GR genes appeared ubiquitously expressed, although they showed tissue and region-specific differences in their relative expression. The distribution and regulation by glucocorticoid manipulations of total GR mRNA in hippocampus, liver and thymus was consistent with that shown in previous studies, apart from in hippocampus where a novel observation of upregulation of GR mRNA levels by adrenalectomy with supraphysiological glucocorticoid replacement was made. However, the observed changes in total GR mRNA in hippocampus, liver and thymus could not be accounted for by dramatic regulation by glucocorticoids of any single one of the exons 1 studied here, suggesting that glucocorticoid autoregulation of GR levels may regulate the activity of all the promoters equally. Finally, preliminary data suggests that a region of DNase I sensitivity and hence open chromatin structure may be associated with the widely-expressed exon 1_{10} of the GR gene. Further work is required to elucidate the molecular mechanisms of transcriptional control of the GR gene.

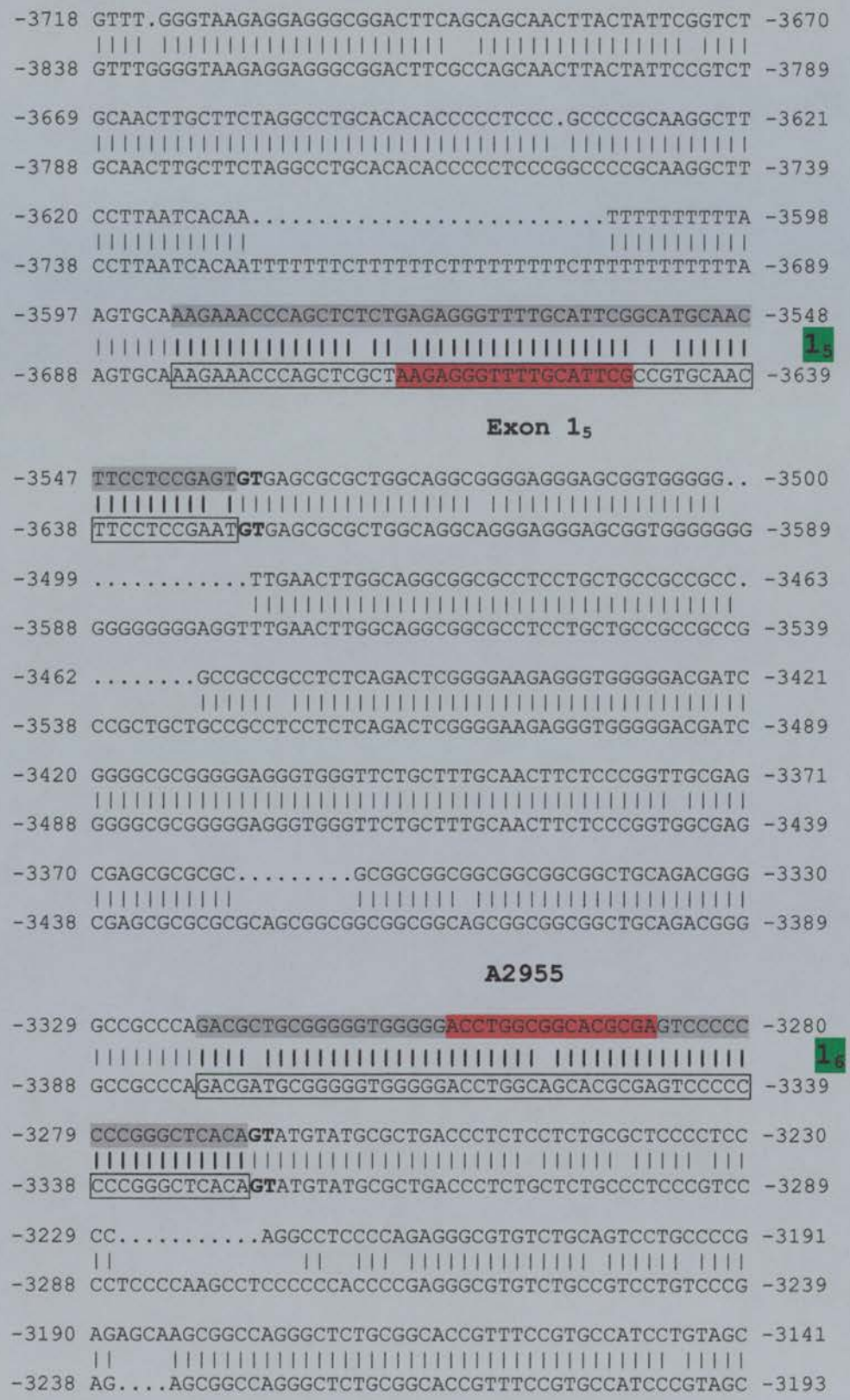
Appendix A: sequences of the rat and mouse GR gene promoter regions

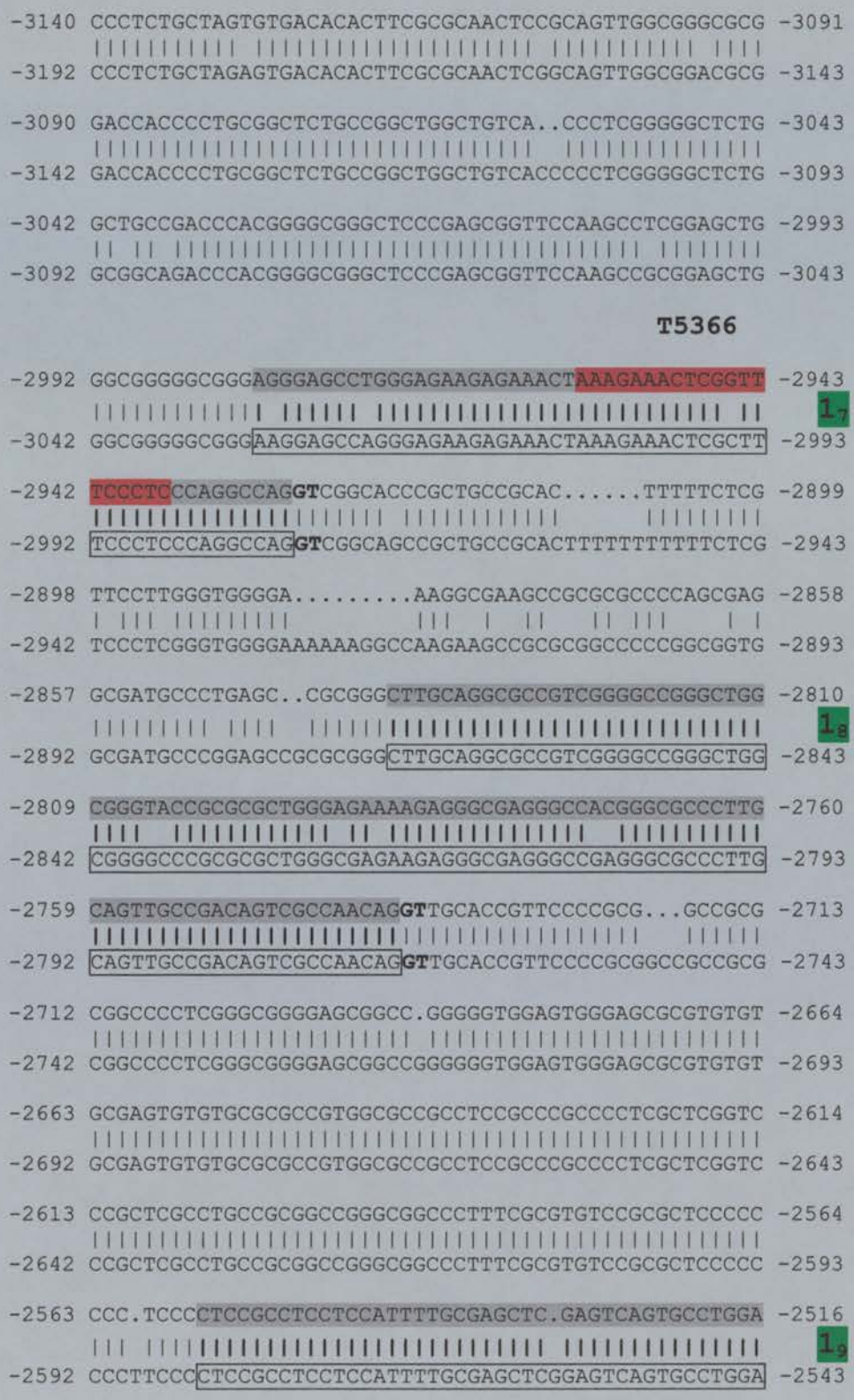
Sequence alignment of rat and mouse GR gene promoter regions using the published rat GR cDNA sequence (MIESFELD et al., 1986) and mouse GR promoter sequence accession number X66367, shown as the upper and lower sequences respectively. Exon 1 sequences isolated from rat hippocampal GR mRNA by 5'-RACE (McCormick et al., 2000) are represented by shaded areas (except for exon 1_6 where the shaded nucleotides are those identified from the rat cDNA sequence (MIESFELD et al., 1986)). Corresponding exons 1 found in the mouse GR promoter sequence are boxed. Splice donor sites and the splice acceptor site 13 nucleotides into exon 2 are shown in bold type.

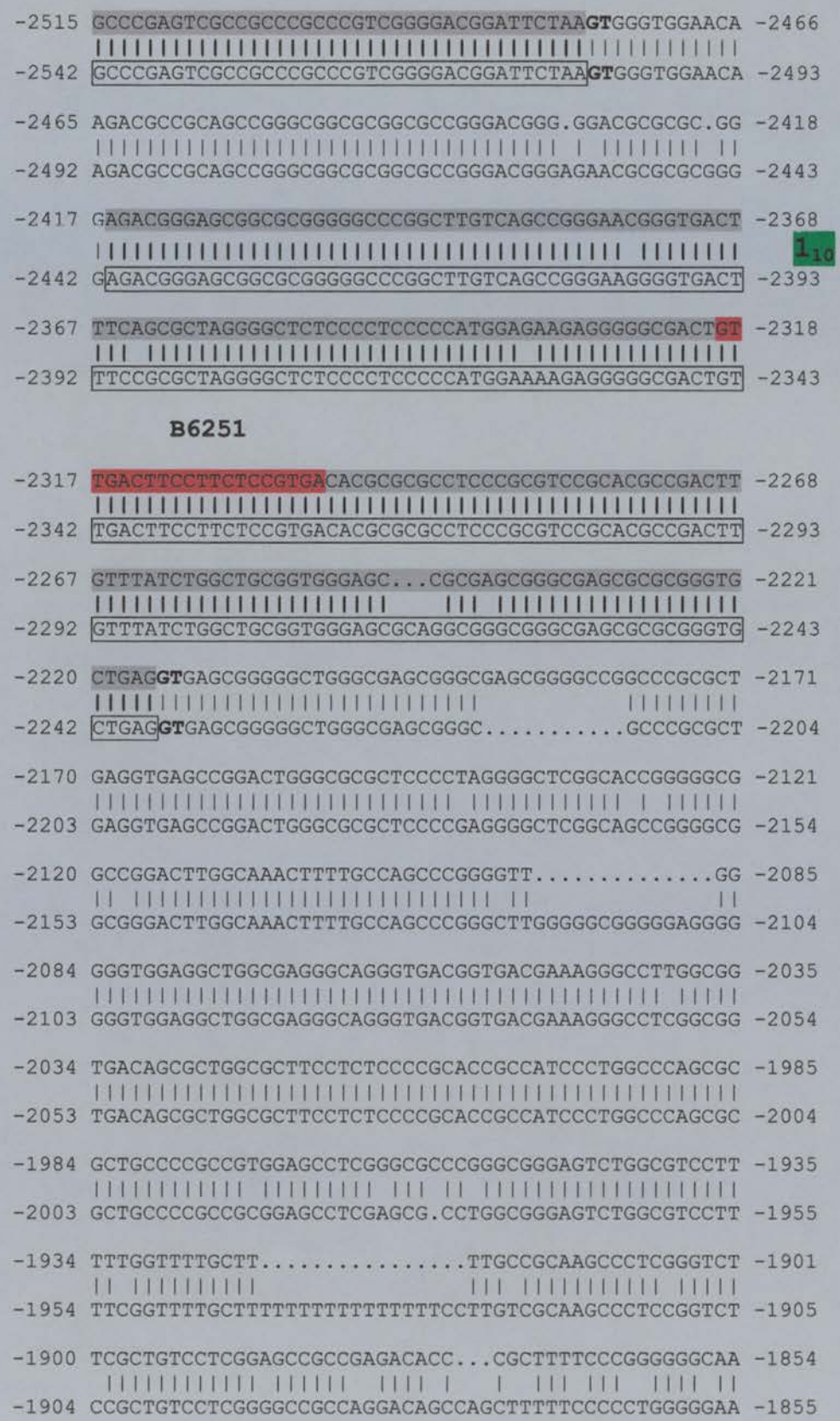
Position of primers for reverse transcriptase PCR (section 2.1.8.1) are indicated by coloured areas. Red areas indicate primer position. Yellow areas indicate regions where two primers overlap.

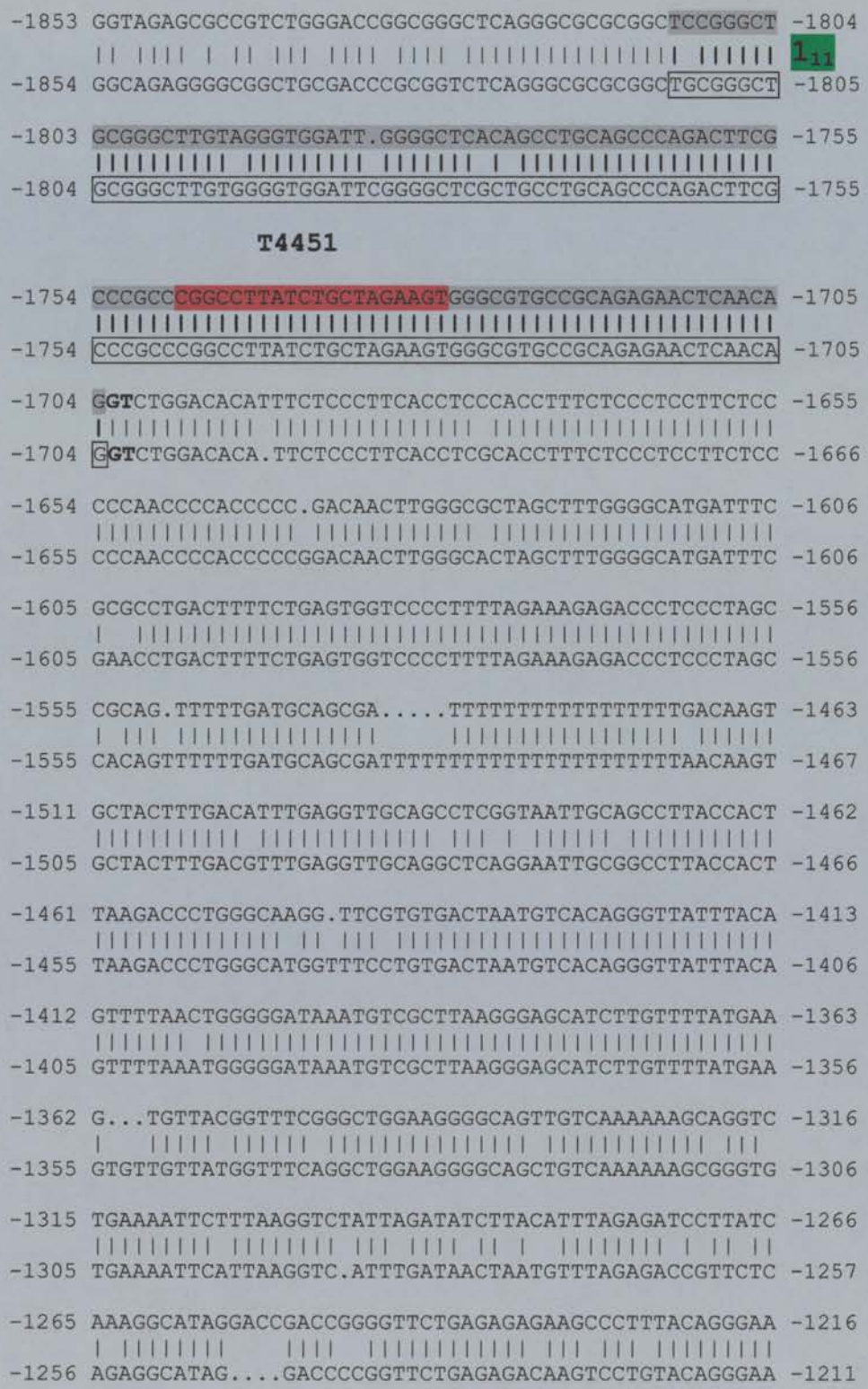
```
-4599 ....TAGTATAGGTTTTCCTTCTTGAGGTATCAAGCTTCTATTCCTTTGC -4554
          | | || |||||||||||||||||| |||||||||   ||||||||
-4721 GCACTGGGATGGGTTTTCCTTCTTGAGGTGTCAAGCTTCGGCTCCTTTGC -4672

-4553 CAAGATGGCTGCCCTGGATCCCATGGAGGTAGCGACCGTGCGGCATCTCT -4504
      ||||||||| ||||||| ||||||||||||||||||  |||| ||| ||||
-4671 CAAGATGGCGGCCCTGGCTCCCATGGAGGTAGCGATTGTGCAGCACCTCT -4622

-4503 GCCCAAGGAGCCCGCTTACAGTCACGTTCTCCCCGTGCAAAGCGGACGAT -4454
      | || ||| |||  ||||||| ||||  || |||| |||||||||| ||
-4621 GGCCCAGGGGCCGTCTTACAGCCACGGCCTACCCGCGCAAAGCGGAAGAC -4572

-4453 ACATTGGGCAGCCTTTAAGCTTTTCATCCAAGAAAGAACGACTCGGGTTT -4404
      ||||||||||||||||   ||| ||||||||||  || ||||| |||
-4571 ACATTGGGCAGCCTTTACATTTTCCATCCAAGAAAGGGCGCCTCGGTTTT -4522

-4403 GACGCCAAAGAGCACCTTTGCCAAGATGGTGACCGTGCGGCGTCACTGCT -4354
      || || |||||||||| |||||| |||||||||||| ||||||||||||
-4521 GAAGCTAAAGAGCACCTCTGCCAAAATGGTGACCGTGTGGCGTCACTGCT -4472
```


-4353 CTTTACCAAGATGGCGGCGAGGGACTTCCGGCACGCGCTTCCCCAATCAG -4304
-4471 CTTTACCAAGATGGCGGCGAGAGACTTCCGGCACGCGCTTCCCCAATCAG -4422
-4303 GGATCTCCAAGAGGTCAGGCAGAGGAGACCGCCCTTGGAGTCGAAGTGCG -4254
-4421 AGATCTCCAAGAGGTCAGGCAGAGGAGACCGCCCTCGGAGTCCAAGTGCT -4372
-4253 GCGCGAGCCGCTTCTGCCGCCCGCCGCGGGAGGGCATGAGGTGGAGTCAT -4204
1₄
-4371 GCGCGATCGGCT.....GGGGGAGAGAATGGGGTGGAGCCAC -4335
-4203 GGCCACCTCCGCTCTAATCAGAAGTGCCAAGCGCTGGCACCTGTGGGGGA -4154
-4334 GGCAGCCTCAGCTCCGATCAGAAGTGCCAAGCGCTGGCACCTGTGGGGGA -4285
Exon 1₄
t4449
-4153 GCAAAAGTTACTTCCTTGCACCCCAAAGCAACACCGTAACACCTT..... -4109
-4284 GCAAAAGTTACTTCCTCGCACCCCAAAGCAACACCATAACACCTTACTCC -4235
-4108 ...TCCCGGAGTCCCCCAAATCCTAGCCTATGGCATGAGGTAGAGGGGCA -4062
-4234 CCAACCCCCAGGCCCCTAAACCGTAGCGTGCGGCGCGAGGTAG.GGGGCA -4186
-4061 CGGTCCCGGCGTCGCCCCAGCCTGCCTAACACAGAGGACACCCGAGGGGC -4012
-4185 CGGTCCCAGAGTCGCCCCAGTCTGCC.AAAGGGGCGGGCAGCTTAGGGGC -4136
F5984
-4011 AGGT..ACTGCGCGGCCGCACAGTCA.CCCTACGCCCCTTTCCTGTCCTA -3965
-4136 AGGTCCGCGCGGCGGCTGCACTGTCACCCCCACGCCCCTCTCCTGTCCTA -4087
-3964 GGGGGACCGGCCATGTGTTTCTCTTGGAGACCCGGGGACTCGTATTGGGC -3915
-4086 GGGGGACCGGCCACGTGTTTCTCTTGGAGACCCGGGG.CTCGCCCTGGGA -4038
-3914 ACAGCTGGACGGAGCTAAAAGCTGACGTTTTAAAGATGCATGTTTTTGTT -3865
-4037 ACAGCTGGAGGGAGCTAAACGCTGACGTTGTAAAGATGCGTG.TTTTGTT -3989
-3864 TTATTTGGAGGGACAGAGGGGTCCCTGGAACCCAGAAA....GCTGAGCA -3819
-3988 TTATTTGGAGGGGCAGAGGGGTCCCTGGAACTCAGAAAGAAGGCAGAGCG -3939
-3818 AGGCACCGAGCCTGGAGCAGCAAATGTCAAGATTCGGGGGAGGGGCCTCC -3769
-3938 AGGCACTGAGCCTGGAGCAGCAAATGTCAAGATTTGGGGGAGGGGCCTCC -3889
-3768 GCGGGGAGCTTGGATGCTGGCCCCGAAGGGGGTGGAAGGAGAGGTCAGGA -3719
-3888 GCGGGGAGCTTGGATGCTGGCCCCGAAGGGGGTGGAAGGAGAGGTCAGGA -3839

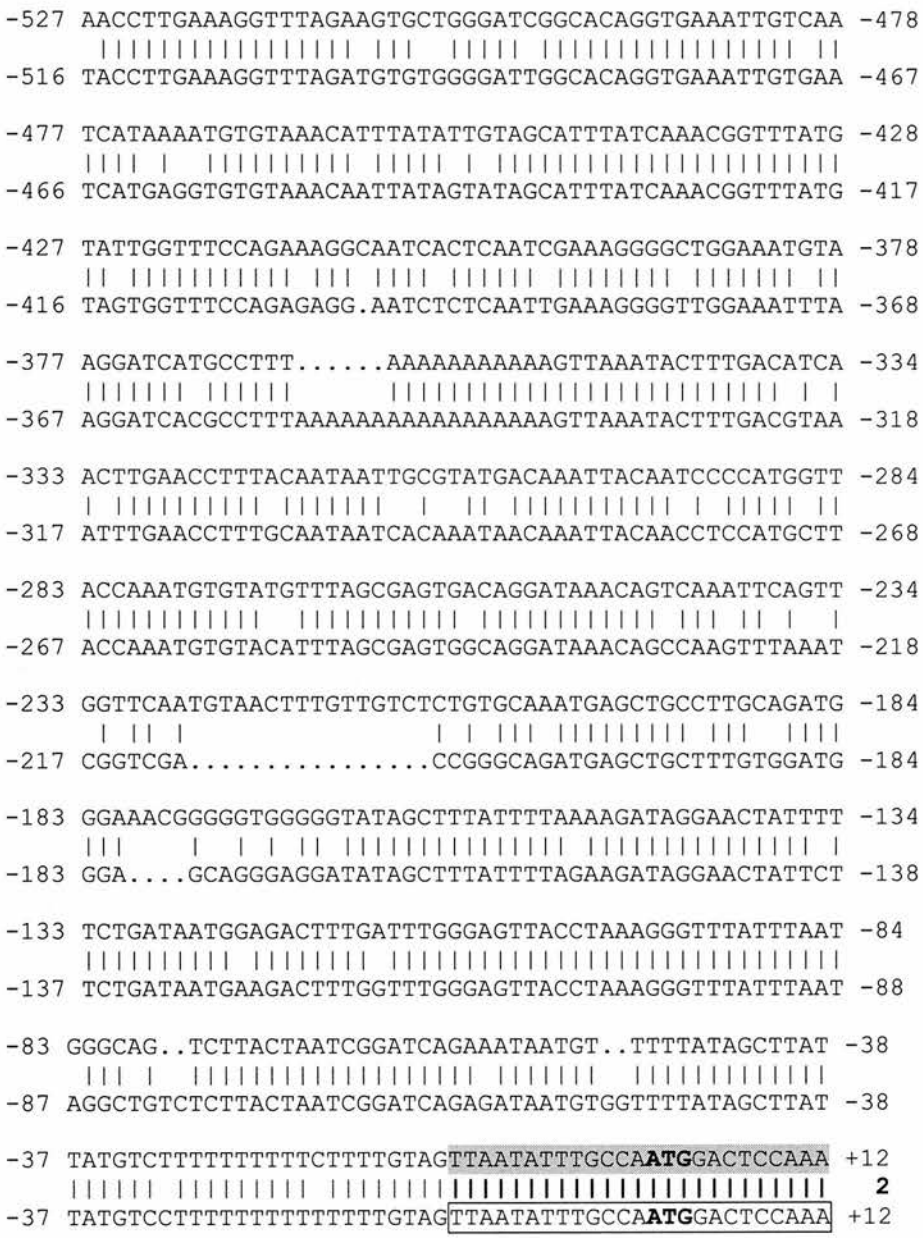

-3718 GTTT.GGGTAAGAGGAGGGCGGACTTCAGCAGCAACTTACTATTCGGTCT -3670
-3838 GTTTGGGGTAAGAGGAGGGCGGACTTCGCCAGCAACTTACTATTCCGTCT -3789
-3669 GCAACTTGCTTCTAGGCCTGCACACACCCCCTCCC.GCCCCGCAAGGCTT -3621
-3788 GCAACTTGCTTCTAGGCCTGCACACACCCCCTCCCGGCCCCGCAAGGCTT -3739
-3620 CCTTAATCACAA.....TTTTTTTTTTA -3598
-3738 CCTTAATCACAATTTTTTTCTTTTTTCTTTTTTTTTCTTTTTTTTTTTTA -3689
-3597 AGTGCAAAGAAACCCAGCTCTCTGAGAGGGTTTTGCATTCGGCATGCAAC -3548
1 5
-3688 AGTGCAAAGAAACCCAGCTCGCTAAGAGGGTTTTGCATTCGCCGTGCAAC -3639
Exon 1 5
-3547 TTCCTCCGAGTGTGAGCGCGCTGGCAGGCGGGGAGGGAGCGGTGGGGG.. -3500
-3638 TTCCTCCGAATGTGAGCGCGCTGGCAGGCAGGGAGGGAGCGGTGGGGGGG -3589
-3499 .....TTGAACTTGGCAGGCGGCGCCTCCTGCTGCCGCCGCC. -3463
-3588 GGGGGGGGAGGTTTGAACTTGGCAGGCGGCGCCTCCTGCTGCCGCCGCCG -3539
-3462 .....GCCGCCGCCTCTCAGACTCGGGGAAGAGGGTGGGGGACGATC -3421
-3538 CCGCTGCTGCCGCCTCCTCTCAGACTCGGGGAAGAGGGTGGGGGACGATC -3489
-3420 GGGGCGCGGGGGAGGGTGGGTTCTGCTTTGCAACTTCTCCCGGTTGCGAG -3371
-3488 GGGGCGCGGGGGAGGGTGGGTTCTGCTTTGCAACTTCTCCCGGTGGCGAG -3439
-3370 CGAGCGCGCGC.....GCGGCGGCGGCGGCGGCGGCTGCAGACGGG -3330
-3438 CGAGCGCGCGCGCAGCGGCGGCGGCGGCAGCGGCGGCGGCTGCAGACGGG -3389
A2955
-3329 GCCGCCCAGACGCTGCGGGGGTGGGGGACCTGGCGGCACGCGAGTCCCCC -3280
1 6
-3388 GCCGCCCAGACGATGCGGGGGTGGGGGACCTGGCAGCACGCGAGTCCCCC -3339
-3279 CCCGGGCTCACAGTATGTATGCGCTGACCCTCTCCTCTGCGCTCCCCTCC -3230
-3338 CCCGGGCTCACAGTATGTATGCGCTGACCCTCTGCTCTGCCCTCCCGTCC -3289
-3229 CC.....AGGCCTCCCCAGAGGGCGTGTCTGCAGTCCTGCCCCG -3191
-3288 CCTCCCCAAGCCTCCCCCCACCCCGAGGGCGTGTCTGCCGTCCTGTCCCG -3239
-3190 AGAGCAAGCGGCCAGGGCTCTGCGGCACCGTTTCCGTGCCATCCTGTAGC -3141
-3238 AG....AGCGGCCAGGGCTCTGCGGCACCGTTTCCGTGCCATCCCGTAGC -3193


T5366
1 7
1 8
1 9


-2515 GCCCGAGTCGCCGCCCGCCCGTCGGGGACGGATTCTAAGTGGGTGGAACA -2466
-2542 GCCCGAGTCGCCGCCCGCCCGTCGGGGACGGATTCTAAGTGGGTGGAACA -2493
-2465 AGACGCCGCAGCCGGGCGGCGCGGCGCCGGGACGGG.GGACGCGCGC.GG -2418
-2492 AGACGCCGCAGCCGGGCGGCGCGGCGCCGGGACGGGAGAACGCGCGCGGG -2443
-2417 GAGACGGGAGCGGCGCGGGGGCCCGGCTTGTCAGCCGGGAACGGGTGACT -2368
1 10
-2442 GAGACGGGAGCGGCGCGGGGGCCCGGCTTGTCAGCCGGGAAGGGGTGACT -2393
-2367 TTCAGCGCTAGGGGCTCTCCCCTCCCCCATGGAGAAGAGGGGGCGACTGT -2318
-2392 TTCCGCGCTAGGGGCTCTCCCCTCCCCCATGGAAAAGAGGGGGCGACTGT -2343
B6251
-2317 TGACTTCCTTCTCCGTGACACGCGCGCCTCCCGCGTCCGCACGCCGACTT -2268
-2342 TGACTTCCTTCTCCGTGACACGCGCGCCTCCCGCGTCCGCACGCCGACTT -2293
-2267 GTTTATCTGGCTGCGGTGGGAGC...CGCGAGCGGGCGAGCGCGCGGGTG -2221
-2292 GTTTATCTGGCTGCGGTGGGAGCGCAGGCGGGCGGGCGAGCGCGCGGGTG -2243
-2220 CTGAGGTGAGCGGGGGCTGGGCGAGCGGGCGAGCGGGGCCGGCCCGCGCT -2171
-2242 CTGAGGTGAGCGGGGGCTGGGCGAGCGGGC.....GCCCGCGCT -2204
-2170 GAGGTGAGCCGGACTGGGCGCGCTCCCCTAGGGGCTCGGCACCGGGGGCG -2121
-2203 GAGGTGAGCCGGACTGGGCGCGCTCCCCGAGGGGCTCGGCAGCCGGGGCG -2154
-2120 GCCGGACTTGGCAAACTTTTGCCAGCCCGGGGTT.....GG -2085
-2153 GCGGGACTTGGCAAACTTTTGCCAGCCCGGGCTTGGGGGCGGGGGAGGGG -2104
-2084 GGGTGGAGGCTGGCGAGGGCAGGGTGACGGTGACGAAAGGGCCTTGGCGG -2035
-2103 GGGTGGAGGCTGGCGAGGGCAGGGTGACGGTGACGAAAGGGCCTCGGCGG -2054
-2034 TGACAGCGCTGGCGCTTCCTCTCCCCGCACCGCCATCCCTGGCCCAGCGC -1985
-2053 TGACAGCGCTGGCGCTTCCTCTCCCCGCACCGCCATCCCTGGCCCAGCGC -2004
-1984 GCTGCCCCGCCGTGGAGCCTCGGGCGCCCGGGCGGGAGTCTGGCGTCCTT -1935
-2003 GCTGCCCCGCCGCGGAGCCTCGAGCG.CCTGGCGGGAGTCTGGCGTCCTT -1955
-1934 TTTGGTTTTGCTT.....TTGCCGCAAGCCCTCGGGTCT -1901
-1954 TTCGGTTTTGCTTTTTTTTTTTTTTTCCTTGTCGCAAGCCCTCCGGTCT -1905
-1900 TCGCTGTCCTCGGAGCCGCCGAGACACC...CGCTTTTCCCGGGGGGCAA -1854
-1904 CCGCTGTCCTCGGGGCCGCCAGGACAGCCAGCTTTTTCCCCCTGGGGGAA -1855


-1853 GGTAGAGCGCCGTCTGGGACCGGCGGGCTCAGGGCGCGCGGCTCCGGGCT -1804
1 11
-1854 GGCAGAGGGGCGGCTGCGACCCGCGGTCTCAGGGCGCGCGGCTGCGGGCT -1805
-1803 GCGGGCTTGTAGGGTGGATT.GGGGCTCACAGCCTGCAGCCCAGACTTCG -1755
-1804 GCGGGCTTGTGGGGTGGATTCGGGGCTCGCTGCCTGCAGCCCAGACTTCG -1755
T4451
-1754 CCCGCCCGGCCTTATCTGCTAGAAGTGGGCGTGCCGCAGAGAACTCAACA -1705
-1754 CCCGCCCGGCCTTATCTGCTAGAAGTGGGCGTGCCGCAGAGAACTCAACA -1705
-1704 GGTCTGGACACATTTCTCCCTTCACCTCCCACCTTTCTCCCTCCTTCTCC -1655
-1704 GGTCTGGACACA.TTCTCCCTTCACCTCGCACCTTTCTCCCTCCTTCTCC -1666
-1654 CCCAACCCCACCCCC.GACAACTTGGGCGCTAGCTTTGGGGCATGATTTC -1606
-1655 CCCAACCCCACCCCCGGACAACTTGGGCACTAGCTTTGGGGCATGATTTC -1606
-1605 GCGCCTGACTTTTCTGAGTGGTCCCCTTTTAGAAAGAGACCCTCCCTAGC -1556
-1605 GAACCTGACTTTTCTGAGTGGTCCCCTTTTAGAAAGAGACCCTCCCTAGC -1556
-1555 CGCAG.TTTTTGATGCAGCGA.....TTTTTTTTTTTTTTTTTGACAAGT -1463
-1555 CACAGTTTTTTGATGCAGCGATTTTTTTTTTTTTTTTTTTTAACAAGT -1467
-1511 GCTACTTTGACATTTGAGGTTGCAGCCTCGGTAATTGCAGCCTTACCACT -1462
-1505 GCTACTTTGACGTTTGAGGTTGCAGGCTCAGGAATTGCGGCCTTACCACT -1466
-1461 TAAGACCCTGGGCAAGG.TTCGTGTGACTAATGTCACAGGGTTATTTACA -1413
-1455 TAAGACCCTGGGCATGGTTTCCTGTGACTAATGTCACAGGGTTATTTACA -1406
-1412 GTTTTAACTGGGGGATAAATGTCGCTTAAGGGAGCATCTTGTTTTATGAA -1363
-1405 GTTTTAAATGGGGGATAAATGTCGCTTAAGGGAGCATCTTGTTTTATGAA -1356
-1362 G...TGTTACGGTTTCGGGCTGGAAGGGGCAGTTGTCAAAAAAGCAGGTC -1316
-1355 GTGTTGTTATGGTTTCAGGCTGGAAGGGGCAGCTGTCAAAAAAGCGGGTG -1306
-1315 TGAAAATTCTTTAAGGTCTATTAGATATCTTACATTTAGAGATCCTTATC -1266
-1305 TGAAAATTCATTAAGGTC.ATTTGATAACTAATGTTTAGAGACCGTTCTC -1257
-1265 AAAGGCATAGGACCGACCGGGGTTCTGAGAGAGAAGCCCTTTACAGGGAA -1216
-1256 AGAGGCATAG....GACCCCGGTTCTGAGAGACAAGTCCTGTACAGGGAA -1211

```
-1215 GAATCCTAGGGTAGGTTCCA..CCCCTCTCCACCTTCCCTGAATTTCCCT -1168
      ||| |||| || |||| |||  || ||||||||||||    |||| ||||
-1210 GAAGCCTACGGCAGGTCCCAGCCCTCTCTCCACCTTCCTCAAATTCCCCT -1161

-1167 TTCAGAGAAGGTGGTCATACTTAATGTCTTGGTACAGGAAAAGTTTACCA -1118
      | ||||||||  |||||||||| |||||||||||||||||| |||||||
-1160 TCCAGAGAAGAAGGTCATACTTAGTGTCTTGGTACAGGAAAAATTTACCA -1111

-1117 TTGTAT...TGGGGATCCCAAATATATTTGTCATAGTCTTTGCCAGCCCC -1071
      || |||    |||||| ||||||||||||||| |||||||| | |||| |
-1110 TTTTATGGGAGGGGATTCCAAATATATTTGTCGTAGTCTTT.CTAGCCTC -1062

-1070 TCAAAACATTTTGATTATTACTAACATACTAGCAATCTGGAGGAATACAG -1021
      |||||||||||||||||||| ||| || |||||| |||||||||||| |
-1061 TCAAAACATTTTGATTATTAATAATAT.CTAGCAGTCTGGAGGAATA.AA -1014

-1020 TAAAGGTTTAAAACTACAGAGAGTATTTTTTCTGAGCGTTTTCTTGAATG -971
      |||||||||||||| ||||||  ||| | |  |     ||||||||||
-1013 TAAAGGTTTAAAACTGCAGAGAAAATTATCTGAG...CATTTCTTGAATG -967

-970 GGGTTTATTTGAGTTTATATGTGATTTGACTGTCCAGTTTTTCTG.TTTT -922
     || ||||||||| |||||||||||||  || |||||||||||||| ||||
-966 GGTTTTATTTGACTTTATATGTGATTCTACAGTCCAGTTTTTCTGTTTTT -917

-921 CCCGGTATTTACATCTTTGGAAAGAAAAATCTTAAACTTATAGATAAAAT -872
     || |||||||||| |||||||||| ||||||| ||||||||||||||||
-916 CCTGGTATTTACATATTTGGAAAGAGAAATCTTCAACTTATAGATAAAAT -867

-871 ATTTTATACTGAGTATATCAAACAA..TTTTTAAAAAAGAATACAATTCC -824
     ||||| || |||| |||| ||||||  |||| ||||| ||||| || ||
-866 ATTTTGTATTGAGCATATTAAACAAATTTTTAAAAAAGGAATATAACTCA -817

-823 ATAAATCTTGGTGTTAGGAATTTTAATAAGCTTTGCTCTATTACACTATT -774
      |||||||| |||||||||||||| ||||||| || ||||||| ||||||
-816 GTAAATCTTCGTGTTAGGAATTTTGATAAGCTCTGTTCTATTAAACTATT -767

-773 TAAATAGGTTAAAATTATAGTGAAGAAGCCAGTACAAATTCTACTCTGTT -724
     |||||| ||||||||| ||| |||||||||||||| | |||||||| |
-766 TAAATACGTTAAAATTGTAGGGAAGAAGCCAGTACACACTCTACTCTGCT -717

-723 TTTAAAGATATACATTTTAGGCTGTATATAATATCTATAATTTCTTATCT -674
     ||||| |||  ||||||||| |||||||||||| ||| ||||||||| |
-716 TTTAAGGATGCACATTTTAGTCTGTATATAATATATATTATTTCTTATAT -667

-673 CCAAAATTTGAAGGTAGGTGATACTAGACAGGCATATTTATTTGAAAATA -624
     || || |||||||||||||||||||||||||||||||| ||||||||
-666 CCCAAGTTTGAAGGTAGGTGATACTAGACAGGCATATTTAGTTGAAAATG -617

-623 GAGTTTCAAAGTAAGAGCCTTTCCCTAGCTCTACAAGATAAGCAGCTCAG -574
     |||||||| ||||||||| |||||||||| || ||||| ||||||| |||
-616 GAGTTTCAGAGTAAGAGCTTTTCCCTAGCCCTGCAAGACAAGCAGCCCAG -567

-573 CACTGC.TTTTTTTTTCAAAAATAAAAATGTGCATAATTCTT...GGAGG -528
     |||||| |||||||||     |  ||||||||||||||||||   ||  |
-566 CACTGCTTTTTTTTTTTTTTCCAATAAAATGTGCATAATTCTTCAAGGTTG -517
```


-527 AACCTTGAAAGGTTTAGAAGTGCTGGGATCGGCACAGGTGAAATTGTCAA -478
-516 TACCTTGAAAGGTTTAGATGTGTGGGGATTGGCACAGGTGAAATTGTGAA -467
-477 TCATAAAATGTGTAAACATTTATATTGTAGCATTTATCAAACGGTTTATG -428
-466 TCATGAGGTGTGTAAACAATTATAGTATAGCATTTATCAAACGGTTTATG -417
-427 TATTGGTTTCCAGAAAGGCAATCACTCAATCGAAAGGGGCTGGAAATGTA -378
-416 TAGTGGTTTCCAGAGAGG.AATCTCTCAATTGAAAGGGGTTGGAAATTTA -368
-377 AGGATCATGCCTTT......AAAAAAAAAAAGTTAAATACTTTGACATCA -334
-367 AGGATCACGCCTTTAAAAAAAAAAAAAAAAAGTTAAATACTTTGACGTAA -318
-333 ACTTGAACCTTTACAATAATTGCGTATGACAAATTACAATCCCCATGGTT -284
-317 ATTTGAACCTTTGCAATAATCACAAATAACAAATTACAACCTCCATGCTT -268
-283 ACCAAATGTGTATGTTTAGCGAGTGACAGGATAAACAGTCAAATTCAGTT -234
-267 ACCAAATGTGTACATTTAGCGAGTGGCAGGATAAACAGCCAAGTTTAAAT -218
-233 GGTTCAATGTAACTTTGTTGTCTCTGTGCAAATGAGCTGCCTTGCAGATG -184
-217 CGGTCGA.................CCGGGCAGATGAGCTGCTTTGTGGATG -184
-183 GGAAACGGGGGTGGGGGTATAGCTTTATTTTAAAAGATAGGAACTATTTT -134
-183 GGA....GCAGGGAGGATATAGCTTTATTTTAGAAGATAGGAACTATTCT -138
-133 TCTGATAATGGAGACTTTGATTTGGGAGTTACCTAAAGGGTTTATTTAAT -84
-137 TCTGATAATGAAGACTTTGGTTTGGGAGTTACCTAAAGGGTTTATTTAAT -88
-83 GGGCAG..TCTTACTAATCGGATCAGAAATAATGT..TTTTATAGCTTAT -38
-87 AGGCTGTCTCTTACTAATCGGATCAGAGATAATGTGGTTTTATAGCTTAT -38
-37 TATGTCTTTTTTTTTTCTTTTGTAGTTAATATTTGCCAATGGACTCCAAA +12
2
-37 TATGTCCTTTTTTTTTTTTTTGTAGTTAATATTTGCCAATGGACTCCAAA +12

References

Akerblom,I.E., Slater,E.P., Beato,M., Baxter,J.D., and Mellon,P.L. (1988). Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science *241*, 350-353.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, and Watson J.D. (1994). Molecular Biology of the Cell. (New York: Garland Publishing Inc.).

Alexandrova,M. and Farkas,P. (1992). Stress-induced changes of glucocorticoid receptor in rat liver. Journal of Steroid Biochemistry and Molecular Biology *42*, 493-498.

Alexandrova,M., Mascuchova,D., and Tatar,P. (1989). Comparison of the biopotency of corticosterone and dexamethasone acetate in glucocorticoid receptor down regulation in rat liver. Journal of Steroid Biochemistry *32*, 531-535.

Altschul,S.F., Madden,T.L., Schaffer,A.A., Zhang,J., Zhang,Z., Miller,W., and Lipman,D.J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research *25*, 3389-3402.

Andrews,M.H. and Matthews,S.G. (2000). Regulation of glucocorticoid receptor mRNA and heat shock protein 70 mRNA in the developing sheep brain. Brain Research *878*, 174-182.

Anisman,H., Zaharia,M.D., Meaney,M.J., and Merali,Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? International Journal of Developmental Neuroscience *16*, 149-164.

Antakly,T. and Eisen,H.J. (1984). Immunocytochemical localization of glucocorticoid receptor in target cells. Endocrinology *115*, 1984-1989.

Antakly,T., Raquidan,D., Donnell,D., and Katnick,L. (1990). Regulation of glucocorticoid receptor expression: I. Use of a specific radioimmunoassay and antiserum to a synthetic peptide of the N-terminal domain. Endocrinology *126*, 1821-1828.

Antakly,T., Thompson,E.B., and Donnell,D. (1989). Demonstration of the intracellular localization and up-regulation of glucocorticoid receptor by in situ hybridization and immunocytochemistry. Cancer Res *49*, 2230s-2234s.

Arai,M. and Widmaier,E.P. (1993). Steroidogenesis in isolated adrenocortical cells during development in rats. Molecular and Cellular Endocrinology *92*, 91-97.

Arbel,I., Kadar,T., Silbermann,M., and Levy,A. (1994). The effects of long-term corticosterone administration on hippocampal morphology and cognitive performance of middle-aged rats. Brain Research *657*, 227-235.

Archer,T.K., Lefebvre,P., Wolford,R.G., and Hager,G.L. (1992). Transcription factor loading on the MMTV promoter - a bimodal mechanism for promoter activation. Science *255*, 1573-1576.

Argaud,D., Zhang,Q., Pan,W., Maitra,S., Pilkis,S.J., and Lange,A.J. (1996). Regulation of rat liver glucose-6-phosphatase gene expression in different nutritional and hormonal states: gene structure and 5'-flanking sequence. Diabetes *45*, 1563-1571.

Arriza,J.L., Simerly,R.B., Swanson,L.W., and Evans,R.M. (1988). The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron *1*, 887-900.

Ashraf,J., Kunapuli,S., Chilton,D., and Thompson,E.B. (1991). Cortivazol mediated induction of glucocorticoid receptor messenger ribonucleic acid in wild-type and dexamethasone-resistant human leukemic (CEM) cells. J Steroid Biochem Mol Biol *38*, 561-568.

Ashwell,J.D., Lu,F.W., and Vacchio,M.S. (2000). Glucocorticoids in T cell development and function. Annual Review of Immunology *18*, 309-345.

Auphan,N., Didonato,J.A., Rosette,C., Helmberg,A., and Karin,M. (1995). Immunosuppression by glucocorticoids - inhibition of NF-kappa-B activity through induction of I-kappa-B synthesis. Science *270*, 286-290.

Avishai-Eliner,S., Hatalski,C.G., Tabachnik,E., Eghbal-Ahmadi,M., and Baram,T.Z. (1999). Differential regulation of glucocorticoid receptor messenger RNA (GR-mRNA) by maternal deprivation in immature rat hypothalamus and limbic regions. Developmental Brain Research *114*, 265-268.

Ayoubi,T.A.Y. and VanDeVen,W.J.M. (1996). Regulation of gene expression by alternative promoters. Faseb Journal *10*, 453-460.

Ballard,P.L., Ertsey,R., Gonzales,L.W., and Gonzales,J. (1996). Transcriptional regulation of human pulmonary surfactant proteins SP-B and SP-C by glucocorticoids. American Journal of Respiratory Cell and Molecular Biology *14*, 599-607.

Barden,N. (1996). Modulation of glucocorticoid receptor gene expression by antidepressant drugs. Pharmacopsychiatry *29*, 12-22.

Barden,N., Stec,I.S., Montkowski,A., Holsboer,F., and Reul,J.M. (1997). Endocrine profile and neuroendocrine challenge tests in transgenic mice expressing antisense RNA against the glucocorticoid receptor. Neuroendocrinology *66*, 212-220.

Barker,D.J. (1990). The fetal and infant origins of adult disease. British Medical Journal *301*, 1111.

Barker,D.J. (1991). Deprivation in infancy and risk of ischaemic heart disease. Lancet *337*, 981.

Barker,D.J., Bull,A.R., Osmond,C., and Simmonds,S.J. (1990). Fetal and placental size and risk of hypertension in adult life. British Medical Journal *301*, 259-262.

Bauer,A., Tronche,F., Wessely,O., Kellendonk,C., Reichardt,H.M., Steinlein,P., Schutz,G., and Beug,H. (1999). The glucocorticoid receptor is required for stress erythropoiesis. Genes & Development *13*, 2996-3002.

Beato,M. (1989). Gene-Regulation By Steroid-Hormones. Cell *56*, 335-344.

Beato,M., Chavez,S., and Truss,M. (1996). Transcriptional regulation by steroid hormones. Steroids *61*, 240-251.

Belanoff,J.K., Gross,K., Yager,A., and Schatzberg,A.F. (2001). Corticosteroids and cognition. Journal of Psychiatric Research *35*, 127-145.

Berdanier,C.D. (1989). Role of glucocorticoids in the regulation of lipogenesis. Faseb Journal *3*, 2179-2183.

Berger,S., Bleich,M., Schmid,W., Cole,T.J., Peters,J., Watanabe,H., Kriz,W., Warth,R., Greger,R., and Schutz,G. (1998). Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. Proceedings of the National Academy of Sciences of the United States of America *95*, 9424-9429.

Bertagna,X., Bertagna,C., Laudat,M.H., Husson,J.M., Girard,F., and Luton,J.P. (1986). Pituitary-adrenal response to the antiglucocorticoid action of RU 486 in Cushing's syndrome. The Journal of Clinical Endocrinology and Metabolism *63*, 639-643.

Bertorelli,G., Bocchino,V., and Olivieri,D. (1998). Heat shock protein interactions with the glucocorticoid receptor. Pulmonary Pharmacology & Therapeutics *11*, 7-12.

Betancur,C., Borrell,J., and Guaza,C. (1995). Cytokine regulation of corticosteroid receptors in the rat hippocampus: effects of interleukin-1, interleukin-6, tumor necrosis factor and lipopolysaccharide. Neuroendocrinology *62*, 47-54.

Biagini,G., Pich,E.M., Carani,C., Marrama,P., and Agnati,L.F. (1998). Postnatal maternal separation during the stress hyporesponsive period enhances the adrenocortical response to novelty in adult rats by affecting feedback regulation in the CA1 hippocampal field. International Journal of Developmental Neuroscience *16*, 187-197.

Bird,A., Taggart,M., Frommer,M., Miller,O.J., and Macleod,D. (1985). A fraction of the mouse genome that is derived from islands of nonmethylated, CpG-rich DNA. Cell *40*, 91-99.

Bledsoe,R.K., Montana,V.G., Stanley,T.B., Delves,C.J., Apolito,C.J., McKee,D.D., Consler,T.G., Parks,D.J., Stewart,E.L., Willson,T.M., Lambert,M.H., Moore,J.T., Pearce,K.H., and Xu,H.E. (2002). Crystal structure of the glucocorticoid receptor ligand binding domain reveals a novel mode of receptor dimerization and coactivator recognition. Cell *110*, 93-105.

Bluthe,R.M., Walter,V., Parnet,P., Laye,S., Lestage,J., Verrier,D., Poole,S., Stenning,B.E., Kelley,K.W., and Dantzer,R. (1994). Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism. Comptes Rendus de L"Academie Des Sciences.Serie Iii, Sciences de La Vie *317*, 499-503.

Bodnoff,S.R., Humphreys,A.G., Lehman,J.C., Diamond,D.M., Rose,G.M., and Meaney,M.J. (1995). Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. Journal of Neuroscience *15*, 61-69.

Bodwell,J.E., Webster,J.C., Jewell,C.M., Cidlowski,J.A., Hu,J.M., and Munck,A. (1998). Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. Journal of Steroid Biochemistry and Molecular Biology *65*, 91-99.

Borbhuiya,M.A. and Sharma,R. (1995). Regulation of hepatic glucocorticoid receptor during development of mice. Biochemistry and Molecular Biology International *37*, 645-652.

Bradbury,M.J., Akana,S.F., and Dallman,M.F. (1994). Roles Of Type-I And Type-Ii Corticosteroid Receptors In Regulation Of Basal Activity In The Hypothalamo-Pituitary- Adrenal Axis During The Diurnal Trough And The Peak - Evidence For A Nonadditive Effect Of Combined Receptor Occupation. Endocrinology *134*, 1286-1296.

Brandeis,M., Frank,D., Keshet,I., Siegfried,Z., Mendelsohn,M., Nemes,A., Temper,V., Razin,A., and Cedar,H. (1994). Sp1 elements protect a CpG island from de novo methylation. Nature *371*, 435-438.

Breslin,M.B., Geng,C.D., and Vedeckis,W.V. (2001). Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Molecular Endocrinology *15*, 1381-1395.

Breslin,M.B. and Vedeckis,W.V. (1998). The human glucocorticoid receptor promoter upstream sequences contain binding sites for the ubiquitous transcription factor, Yin Yang 1. J Steroid Biochem Mol Biol *67*, 369-381.

Bresnick,E.H., Bustin,M., Marsaud,V., Richard,F., and Hager,G.L. (1992). The transcriptionally-active MMTV promoter is depleted of histone H1. Nucleic Acids Research *20*, 273-278.

Brien,R.M., Lucas,P.C., Forest,C.D., Magnuson,M.A., and Granner,D.K. (1990). Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. Science *249*, 533-537.

Brown,D.H., Sheridan,J., Pearl,D., and Zwilling,B.S. (1993). Regulation of mycobacterial growth by the hypothalamus-pituitary-adrenal axis: differential responses of Mycobacterium bovis BCG-resistant and -susceptible mice. Infection and Immunity *61*, 4793-4800.

Brown,E.S., Rush,A.J., and McEwen,B.S. (1999). Hippocampal remodeling and damage by corticosteroids: implications for mood disorders. Neuropsychopharmacology *21*, 474-484.

Brown,R.C., Cascio,C., and Papadopoulos,V. (2000). Pathways of neurosteroid biosynthesis in cell lines from human brain: regulation of dehydroepiandrosterone formation by oxidative stress and beta-amyloid peptide. Journal of Neurochemistry *74*, 847-859.

Brown,R.W., Diaz,R., Robson,A.C., Kotelevtsev,Y.V., Mullins,J.J., Kaufman,M.H., and Seckl,J.R. (1996). The ontogeny of 11 beta-hydroxysteroid dehydrogenase type 2 and mineralocorticoid receptor gene expression reveal intricate control of glucocorticoid action in development. Endocrinology *137*, 794-797.

Brzozowski,A.M., Pike,A.C., Dauter,Z., Hubbard,R.E., Bonn,T., Engstrom,O., Ohman,L., Greene,G.L., Gustafsson,J.A., and Carlquist,M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. Nature *389*, 753-758.

Burns,K., Duggan,B., Atkinson,E.A., Famulski,K.S., Nemer,M., Bleackley,R.C., and Michalak,M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature *367*, 476-480.

Bush,B.M. (1991). The Endocrine System. In Canine Medicine And Therapeutics, E. A. Chandler, D. J. Thompson, J. B. Sutton, and C. J. Price, eds. (Oxford: Blackwell Scientific Publications), pp. 309-364.

Cameron,H.A. and Gould,E. (1994). Adult neurogenesis is regulated by adrenal steroids in the dentate gyrus. Neuroscience *61*, 203-209.

Cameron,H.A., McEwen,B.S., and Gould,E. (1995). Regulation of adult neurogenesis by excitatory input and NMDA receptor activation in the dentate gyrus. Journal of Neuroscience *15*, 4687-4692.

Cameron,H.A. and McKay,R.D. (1999). Restoring production of hippocampal neurons in old age. Nature Neuroscience *2*, 894-897.

Cameron,H.A., Woolley,C.S., and Gould,E. (1993). Adrenal steroid receptor immunoreactivity in cells born in the adult rat dentate gyrus. Brain Research *611*, 342-346.

Cavenee,W.K. and Melnykovych,G. (1977). Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase in HeLa cells by glucocorticoids. The Journal of Biological Chemistry *252*, 3272-3276.

Chao,H.M., Ma,L.Y., McEwen,B.S., and Sakai,R.R. (1998a). Regulation of glucocorticoid receptor and mineralocorticoid receptor messenger ribonucleic acids by selective agonists in the rat hippocampus. Endocrinology *139*, 1810-1814.

Chao,H.M., Sakai,R.R., Ma,L.Y., and McEwen,B.S. (1998b). Adrenal steroid regulation of neurotrophic factor expression in the rat hippocampus. Endocrinology *139*, 3112-3118.

Chen,F.H., Watson,C.S., and Gametchu,B. (1999a). Association of the glucocorticoid receptor alternatively- spliced transcript 1A with the presence of the high molecular weight membrane glucocorticoid receptor in mouse lymphoma cells. Journal of Cellular Biochemistry *74*, 430-446.

Chen,F.H., Watson,C.S., and Gametchu,B. (1999b). Multiple glucocorticoid receptor transcripts in membrane glucocorticoid receptor-enriched S-49 mouse lymphoma cells. Journal of Cellular Biochemistry *74*, 418-429.

Chretien,S., Dubart,A., Beaupain,D., Raich,N., Grandchamp,B., Rosa,J., Goossens,M., and Romeo,P.H. (1988). Alternative transcription and splicing of the human porphobilinogen deaminase gene result either in tissue-specific or in housekeeping expression. Proceedings of the National Academy of Sciences of the United States of America *85*, 6-10.

Cianetti,L., Di,C., Zappavigna,V., Bottero,L., Boccoli,G., Testa,U., Russo,G., Boncinelli,E., and Peschle,C. (1990). Molecular mechanisms underlying the expression of the human HOX-5.1 gene. Nucleic Acids Research *18*, 4361-4368.

Cole,M.A., Kim,P.J., Kalman,B.A., and Spencer,R.L. (2000). Dexamethasone suppression of corticosteroid secretion: evaluation of the site of action by receptor measures and functional studies. Psychoneuroendocrinology *25*, 151-167.

Cole,T.J., Blendy,J.A., Monaghan,A.P., Krieglstein,K., Schmid,W., Aguzzi,A., Fantuzzi,G., Hummler,E., Unsicker,K., and Schutz,G. (1995a). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell-development and severely retards lung maturation. Genes & Development *9*, 1608-1621.

Cole,T.J., Blendy,J.A., Monaghan,A.P., Schmid,W., Aguzzi,A., and Schutz,G. (1995b). Molecular-genetic analysis of glucocorticoid signaling during mouse development. Steroids *60*, 93-96.

Cole,T.J., Harris,H.J., Hoong,I., Solomon,N., Smith,R., Krozowski,Z., and Fullerton,M.J. (1999). The glucocorticoid receptor is essential for maintaining basal and dexamethasone-induced repression of the murine corticosteroid-binding globulin gene. Molecular and Cellular Endocrinology *154*, 29-36.

Cole,T.J., Myles,K., Purton,J.F., Brereton,P.S., Solomon,N.M., Godfrey,D.I., and Funder,J.W. (2001). GRKO mice express an aberrant dexamethasone-binding glucocorticoid receptor, but are profoundly glucocorticoid resistant. Molecular and Cellular Endocrinology *173*, 193-202.

Collingwood,T.N., Urnov,F.D., and Wolffe,A.P. (1999). Nuclear receptors: coactivators, corepressors and chromatin remodeling in the control of transcription. Journal of Molecular Endocrinology *23*, 255-275.

Compton,M.M. and Cidlowski,J.A. (1986). Rapid in vivo effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. Endocrinology *118*, 38-45.

Conrad,C.D., Lupien,S.J., and McEwen,B.S. (1999). Support for a bimodal role for type II adrenal steroid receptors in spatial memory. Neurobiology of Learning and Memory *72*, 39-46.

Cordingley,M.G., Riegel,A.T., and Hager,G.L. (1987). Steroid-dependent interaction of transcription factors with the inducible promoter of mouse mammary-tumor virus in vivo. Cell *48*, 261-270.

Csaba,G. and Inczefi-Gonda,A. (1992). Benzpyrene exposure at 15 days of prenatal life reduces the binding capacity of thymic glucocorticoid receptors in adulthood. General Pharmacology *23*, 123-124.

Csaba,G. and Inczefi-Gonda,A. (1998). Transgenerational effect of a single neonatal benzpyrene treatment on the glucocorticoid receptor of the rat thymus. Human & Experimental Toxicology *17*, 88-92.

Csaba,G. and Inczefi-Gonda,A. (2001). Similarities and dissimilarities of newborn and adolescent rats in the binding capacity of thymic glucocorticoid receptors. Mechanisms of Ageing and Development *122*, 327-334.

Csaba,G., Inczefi-Gonda,A., and Szeberenyi,S. (1991). Lasting impact of a single benzpyrene treatment in pre-natal and growing age on the thymic glucocorticoid receptors of rats. General Pharmacology *22*, 815-818.

Cullinan,W.E. and Wolfe,T.J. (2000). Chronic stress regulates levels of mRNA transcripts encoding beta subunits of the GABA(A) receptor in the rat stress axis. Brain Research *887*, 118-124.

Cupps,T.R. and Fauci,A.S. (1982). Corticosteroid-mediated immunoregulation in man. Immunological Reviews *65*, 133-155.

Cyr,M., Charbonneau,C., Morissette,M., Rochford,J., Barden,N., and DiPaolo,T. (2001). Central 5-hydroxytryptamine-2A receptor expression in transgenic mice bearing a glucocorticoid receptor antisense. Neuroendocrinology *73*, 37-45.

Dachir,S., Kadar,T., Robinzon,B., and Levy,A. (1993). Cognitive deficits induced in young rats by long-term corticosterone administration. Behavioral and Neural Biology *60*, 103-109.

Dahlman-Wright, Baumann,H., McEwan,I.J., Almlof,T., Wright,A.P., Gustafsson,J.A., and Hard,T. (1995). Structural characterization of a minimal functional transactivation domain from the human glucocorticoid receptor. Proceedings of the National Academy of Sciences of the United States of America *92*, 1699-1703.

De Bosscher,K., Vanden Berghe,W., and Haegeman,G. (2000). Mechanisms of anti-inflammatory action and of immunosuppression by glucocorticoids: negative interference of activated glucocorticoid receptor with transcription factors. Journal of Neuroimmunology *109*, 16-22.

de Quervain,D.J.F., Roozendaal,B., and McGaugh,J.L. (1998). Stress and glucocorticoids impair retrieval of long-term spatial memory. Nature *394*, 787-790.

Dedhar,S., Rennie,P.S., Shago,M., Hagesteijn,C.Y., Yang,H., Filmus,J., Hawley,R.G., Bruchovsky,N., Cheng,H., and Matusik,R.J. (1994). Inhibition of nuclear hormone receptor activity by calreticulin. Nature *367*, 480-483.

deKloet,E.R., Wallach,G., and McEwen,B.S. (1975). Differences in corticosterone and dexamethasone binding to rat brain and pituitary. Endocrinology *96*, 598-609.

Denton,R.R., Eisen,L.P., Elsasser,M.S., and Harmon,J.M. (1993). Differential autoregulation of glucocorticoid receptor expression in human T- and B-cell lines. Endocrinology *133*, 248-256.

DeRijk,R., Michelson,D., Karp,B., Petrides,J., Galliven,E., Deuster,P., Paciotti,G., Gold,P.W., and Sternberg,E.M. (1997). Exercise and circadian rhythm-induced variations in plasma cortisol differentially regulate interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor-alpha (TNF alpha) production in humans: high sensitivity of TNF alpha and resistance of IL-6. The Journal of Clinical Endocrinology and Metabolism *82*, 2182-2191.

Deroo,B.J. and Archer,T.K. (2001). Glucocorticoid receptor-mediated chromatin remodeling in vivo. Oncogene *20*, 3039-3046.

Desimone,V. and Cortese,R. (1992). Transcription factors and liver-specific genes. Biochimica et Biophysica Acta *1132*, 119-126.

Deuschle,M., Schweiger,U., Weber,B., Gotthardt,U., Korner,A., Schmider,J., Standhardt,H., Lammers,C.H., and Heuser,I. (1997). Diurnal activity and pulsatility of the hypothalamus-pituitary-adrenal system in male depressed patients and healthy controls. The Journal of Clinical Endocrinology and Metabolism *82*, 234-238.

Deuschle,M., Weber,B., Colla,M., Muller,M., Kniest,A., and Heuser,I. (1998). Mineralocorticoid receptor also modulates basal activity of hypothalamus-pituitary-adrenocortical system in humans. Neuroendocrinology *68*, 355-360.

Dhabhar,F.S., McEwen,B.S., and Spencer,R.L. (1993). Stress-response, adrenal-steroid receptor levels and corticosteroid-binding globulin levels - a comparison between Sprague-Dawley, Fischer-344 and Lewis rats. Brain Research *616*, 89-98.

Dhabhar,F.S., Satoskar,A.R., Bluethmann,H., David,J.R., and McEwen,B.S. (2000). Stress-induced enhancement of skin immune function: A role for gamma interferon. Proceedings of the National Academy of Sciences of the United States of America *97*, 2846-2851.

Diorio,D., Viau,V., and Meaney,M.J. (1993). The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. Journal of Neuroscience *13*, 3839-3847.

Djordjevic-Markovic,R., Radic,O., Jelic,V., Radojcic,M., Rapic-Otrin,V., Ruzdijic,S., Krstic-Demonacos,M., Kanazir,S., and Kanazir,D. (1999). Glucocorticoid receptors in ageing rats. Experimental Gerontology *34*, 971-982.

Dong,Y., Aronsson,M., Gustafsson,J.A., and Okret,S. (1989). The mechanism of cAMP-induced glucocorticoid receptor expression. Correlation to cellular glucocorticoid response. Journal of Biological Chemistry *264*, 13679-13683.

Dong,Y., Poellinger,L., Gustafsson,J.A., and Okret,S. (1988). Regulation of glucocorticoid receptor expression - evidence for transcriptional and posttranslational mechanisms. Molecular Endocrinology *2*, 1256-1264.

Drolet,G., Dumont,E.C., Gosselin,I., Kinkead,R., Laforest,S., and Trottier,J.F. (2001). Role of endogenous opioid system in the regulation of the stress response. Progress in Neuro-Psychopharmacology & Biological Psychiatry *25*, 729-741.

Drouin,J., Charron,J., Gagner,J.P., Jeannotte,L., Nemer,M., Plante,R.K., and Wrange,O. (1987). Pro-opiomelanocortin gene: a model for negative regulation of transcription by glucocorticoids. Journal of Cellular Biochemistry *35*, 293-304.

Drouin,J., Sun,Y.L., Chamberland,M., Gauthier,Y., DeLean,A., Nemer,M., and Schmidt,T.J. (1993). Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. Embo Journal *12*, 145-156.

Dubrovsky,B.O., Liquornik,M.S., Noble,P., and Gijsbers,K. (1987). Effects of 5 alpha-dihydrocorticosterone on evoked responses and long-term potentiation. Brain Research Bulletin *19*, 635-638.

Dunn,J.F., Nisula,B.C., and Rodbard,D. (1981). Transport of steroid hormones: binding of 21 endogenous steroids to both testosterone-binding globulin and corticosteroid-binding globulin in human plasma. The Journal of Clinical Endocrinology and Metabolism *53*, 58-68.

Edwards,C.R.W., Benediktsson,R., Lindsay,R.S., and Seckl,J.R. (1993). Dysfunction of placental glucocorticoid barrier - link between fetal environment and adult hypertension. Lancet *341*, 355-357.

Eggert,M., Mows,C.C., Tripier,D., Arnold,R., Michel,J., Nickel,J., Schmidt,S., Beato,M., and Renkawitz,R. (1995). A fraction enriched in a novel glucocorticoid receptor-interacting protein stimulates receptor-dependent transcription in vitro. Journal of Biological Chemistry *270*, 30755-30759.

Ehlert,U., Gaab,J., and Heinrichs,M. (2001). Psychoneuroendocrinological contributions to the etiology of depression, posttraumatic stress disorder, and stress-related bodily disorders: the role of the hypothalamus-pituitary-adrenal axis. Biological Psychology *57*, 141-152.

Eisen,L.P., Elsasser,M.S., and Harmon,J.M. (1988). Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. J Biol Chem *263*, 12044-12048.

Elenkov,I.J. and Chrousos,G.P. (1999). Stress hormones, TH1/TH2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. Trends in Endocrinology and Metabolism *10*, 359-368.

Encio,I.J. and Detera-Wadleigh,S.D. (1991). The genomic structure of the human glucocorticoid receptor. Journal of Biological Chemistry *266*, 7182-7188.

Erdeljan,P., MacDonald,J.F., and Matthews,S.G. (2001). Glucocorticoids and serotonin alter glucocorticoid receptor (GR) but not mineralocorticoid receptor (MR) mRNA levels in fetal mouse hippocampal neurons, in vitro. Brain Research *896*, 130-136.

Evans,S.J., Moore,F.L., and Murray,T.F. (1998). Solubilization and pharmacological characterization of a glucocorticoid membrane receptor from an amphibian brain. Journal of Steroid Biochemistry and Molecular Biology *67*, 1-8.

Faisst,S. and Meyer,S. (1992). Compilation Of Vertebrate-Encoded Transcription Factors. Nucleic Acids Research *20*, 3-26.

Feldman,D., Funder,J.W., and Edelman,I.S. (1973). Evidence for a new class of corticosterone receptors in the rat kidney. Endocrinology *92*, 1429-1441.

Feldman,D. and Loose,D. (1977). Glucocorticoid receptors in adipose tissue. Endocrinology *100*, 398-405.

Feldman,S., Conforti,N., and Melamed,E. (1987). Paraventricular nucleus serotonin mediates neurally stimulated adrenocortical secretion. Brain Research Bulletin *18*, 165-168.

Feldman,S., Conforti,N., and Melamed,E. (1988). Hypothalamic norepinephrine mediates limbic effects on adrenocortical secretion. Brain Research Bulletin *21*, 587-590.

Feldman,S., Newman,M.E., and Weidenfeld,J. (2000). Effects of adrenergic and serotonergic agonists in the amygdala on the hypothalamo-pituitary-adrenocortical axis. Brain Research Bulletin *52*, 531-536.

Feldman,S. and Weidenfeld,J. (1999). Glucocorticoid receptor antagonists in the hippocampus modify the negative feedback following neural stimuli. Brain Research *821*, 33-37.

Felszeghy,K., Gaspar,E., and Nyakas,C. (1996). Long-term selective down-regulation of brain glucocorticoid receptors after neonatal dexamethasone treatment in rats. Journal of Neuroendocrinology *8*, 493-499.

Ferrari,P., Obeyesekere,V.R., Li,K., Wilson,R.C., New,M.I., Funder,J.W., and Krozowski,Z.S. (1996). Point mutations abolish 11 beta-hydroxysteroid dehydrogenase type II activity in three families with the congenital syndrome of apparent mineralocorticoid excess. Molecular and Cellular Endocrinology *119*, 21-24.

Ferrini,M., Magarinos,A.M., and De,N. (1990). Oestrogens down-regulate type I but not type II adrenal corticoid receptors in rat anterior pituitary. Journal of Steroid Biochemistry *35*, 671-677.

Finotto,S., Krieglstein,K., Schober,A., Deimling,F., Lindner,K., Bruhl,B., Beier,K., Metz,J., Garcia-Arraras,J.E., Roig-Lopez,J.L., Monaghan,P., Schmid,W., Cole,T.J., Kellendonk,C., Tronche,F., Schutz,G., and Unsicker,K. (1999). Analysis of mice carrying targeted mutations of the glucocorticoid receptor gene argues against an essential role of glucocorticoid signalling for generating adrenal chromaffin cells. Development *126*, 2935-2944.

Fleig,W.E., Nother-Fleig,G., Steudter,S., Enderle,D., and Ditschuneit,H. (1985). Regulation of insulin binding and glycogenesis by insulin and dexamethasone in cultured rat hepatocytes. Biochimica et Biophysica Acta *847*, 352-361.

Fletcher,T.M., Ryu,B.W., Baumann,C.T., Warren,B.S., Fragoso,G., John,S., and Hager,G.L. (2000). Structure and dynamic properties of a glucocorticoid receptor-induced chromatin transition. Molecular and Cellular Biology *20*, 6466-6475.

Flood,J.F., Vidal,D., Bennett,E.L., Orme,A.E., Vasquez,S., and Jarvik,M.E. (1978). Memory facilitating and anti-amnesic effects of corticosteroids. Pharmacology, Biochemistry, and Behavior *8*, 81-87.

Flouriot,G., Griffin,C., Kenealy,M., Sonntag-Buck,V., and Gannon,F. (1998). Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. Molecular Endocrinology *12*, 1939-1954.

Ford,J., McEwan,I.J., Wright,A.P., and Gustafsson,J.A. (1997). Involvement of the transcription factor IID protein complex in gene activation by the N-terminal transactivation domain of the glucocorticoid receptor in vitro. Molecular Endocrinology *11*, 1467-1475.

Friedman,J.E., Yun,J.S., Patel,Y.M., McGrane,M.M., and Hanson,R.W. (1993). Glucocorticoids regulate the induction of phosphoenolpyruvate carboxykinase (GTP) gene transcription during diabetes. Journal of Biological Chemistry *268*, 12952-12957.

Fujikawa,T., Soya,H., Fukuoka,H., Alam,K.S., Yoshizato,H., McEwen,B.S., and Nakashima,K. (2000). A biphasic regulation of receptor mRNA expressions for growth hormone, glucocorticoid and mineralocorticoid in the rat dentate gyrus during acute stress. Brain Research *874*, 186-193.

Fuxe,K., Wikstrom,A.C., Okret,S., Agnati,L.F., Harfstrand,A., Yu,Z.Y., Granholm,L., Zoli,M., Vale,W., and Gustafsson,J.A. (1985). Mapping of glucocorticoid receptor immunoreactive neurons in the rat tel- and diencephalon using a monoclonal antibody against rat liver glucocorticoid receptor. Endocrinology *117*, 1803-1812.

Gaal,A. and Csaba,G. (1998). Effect of retinoid (vitamin a or retinoic acid) treatment (hormonal imprinting) through breastmilk on the glucocorticoid receptor and estrogen receptor binding capacity of the adult rat offspring. Human & Experimental Toxicology *17*, 560-563.

Galigniana,M.D., Scruggs,J.L., Herrington,J., Welsh,M.J., Carter-Su,C., Housley,P.R., and Pratt,W.B. (1998). Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. Molecular Endocrinology *12*, 1903-1913.

Gametchu,B., Chen,F., Sackey,F., Powell,C., and Watson,C.S. (1999). Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. Steroids *64*, 107-119.

Ganong,W.F. (1989). The Adrenal Medulla & Adrenal Cortex. In Review of Medical Physiology, Appleton & Lange), pp. 301-325.

Ganss,R., Weih,F., and Schutz,G. (1994). The cyclic adenosine 3',5'-monophosphate- and the glucocorticoid-dependent enhancers are targets for insulin repression of tyrosine aminotransferase gene transcription. Molecular Endocrinology *8*, 895-903.

Garcia-Segura,L., Chowen,J.A., and Naftolin,F. (1996). Endocrine glia: roles of glial cells in the brain actions of steroid and thyroid hormones and in the regulation of hormone secretion. Frontiers in Neuroendocrinology *17*, 180-211.

Garvie,C.W. and Wolberger,C. (2001). Recognition of specific DNA sequences. Molecular Cell *8*, 937-946.

Garvin,A.M., Abraham,K.M., Forbush,K.A., Farr,A.G., Davison,B.L., and Perlmutter,R.M. (1990). Disruption of thymocyte development and lymphomagenesis induced by SV40 T-antigen. International Immunology *2*, 173-180.

Gass,P., Kretz,O., Wolfer,D.P., Berger,S., Tronche,F., Reichardt,H.M., Kellendonk,C., Lipp,H.P., Schmid,W., and Schutz,G. (2000). Genetic disruption of mineralocorticoid receptor leads to impaired neurogenesis and granule cell degeneration in the hippocampus of adult mice. EMBO Reports *1*, 447-451.

Gautier,C., Husson,A., and Vaillant,R. (1977). [Effects of glucocorticosteroids on enzymatic activity in the urea cycle in fetal rat liver]. Biochimie *59*, 91-95.

Gearing,K.L., Cairns,W., Okret,S., and Gustafsson,J.A. (1993). Heterogeneity in the 5' untranslated region of the rat glucocorticoid receptor messenger-RNA. Journal of Steroid Biochemistry and Molecular Biology *46*, 635-639.

Genetics Computer Group. Program Manual for the GCG Package. [Version 7]. 1991. 575 Science Drive, Madison, Wisconsin, USA 53711.
Ref Type: Computer Program

Gerlach,J.L. and McEwen,B.S. (1972). Rat brain binds adrenal steroid hormone: radioautography of hippocampus with corticosterone. Science *175*, 1133-1136.

Giguere,V., Hollenberg,S.M., Rosenfeld,M.G., and Evans,R.M. (1986). Functional domains of the human glucocorticoid receptor. Cell *46*, 645-652.

Glaser,R. and Kiecolt,G. (1998). Stress-associated immune modulation: relevance to viral infections and chronic fatigue syndrome. The American Journal of Medicine *105*, 35S-42S.

Godfrey,D.I., Purton,J.F., Boyd,R.L., and Cole,T.J. (2000). Stress-free T-cell development: glucocorticoids are not obligatory. Immunology Today *21*, 606-611.

Godowski,P.J., Rusconi,S., Miesfeld,R., and Yamamoto,K.R. (1987). Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature *325*, 365-368.

Goland,R.S., Jozak,S., Warren,W.B., Conwell,I.M., Stark,R.I., and Tropper,P.J. (1993). Elevated levels of umbilical cord plasma corticotropin-releasing hormone in growth-retarded fetuses. The Journal of Clinical Endocrinology and Metabolism *77*, 1174-1179.

Gomez,S., Zhou,M.Y., Cozza,E.N., Morita,H., Eddleman,F.C., and Gomez,S. (1996). Corticosteroid synthesis in the central nervous system. Endocrine Research *22*, 463-470.

Gould,E., Cameron,H.A., and McEwen,B.S. (1994). Blockade of NMDA receptors increases cell death and birth in the developing rat dentate gyrus. The Journal of Comparative Neurology *340*, 551-565.

Gould,E., Frankfurt,M., Westlind,D., and McEwen,B.S. (1990a). Developing forebrain astrocytes are sensitive to thyroid hormone. Glia *3*, 283-292.

Gould,E., Reeves,A.J., Fallah,M., Tanapat,P., Gross,C.G., and Fuchs,E. (1999). Hippocampal neurogenesis in adult Old World primates. Proceedings of the National Academy of Sciences of the United States of America *96*, 5263-5267.

Gould,E. and Tanapat,P. (1999). Stress and hippocampal neurogenesis. Biol Psychiatry *46*, 1472-1479.

Gould,E., Woolley,C.S., and McEwen,B.S. (1990b). Short-term glucocorticoid manipulations affect neuronal morphology and survival in the adult dentate gyrus. Neuroscience *37*, 367-375.

Green,S. and Chambon,P. (1988). Nuclear receptors enhance our understanding of transcription regulation. Trends in Genetics *4*, 309-314.

Guhaniyogi,J. and Brewer,G. (2001). Regulation of mRNA stability in mammalian cells. Gene *265*, 11-23.

Hadden,J.W. (1992). Thymic Endocrinology. International Journal of Immunopharmacology *14*, 345-352.

Hadoke,P.W., Christy,C., Kotelevtsev,Y.V., Williams,B.C., Kenyon,C.J., Seckl,J.R., Mullins,J.J., and Walker,B.R. (2001). Endothelial cell dysfunction in mice after transgenic knockout of type 2, but not type 1, 11beta-hydroxysteroid dehydrogenase. Circulation *104*, 2832-2837.

Hales,C.N., Barker,D.J., Clark,P.M., Cox,L.J., Fall,C., Osmond,C., and Winter,P.D. (1991). Fetal and infant growth and impaired glucose tolerance at age 64. British Medical Journal *303*, 1019-1022.

Hammond,G.L., Smith,C.L., Paterson,N.A., and Sibbald,W.J. (1990). A role for corticosteroid-binding globulin in delivery of cortisol to activated neutrophils. The Journal of Clinical Endocrinology and Metabolism *71*, 34-39.

Hard,T., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedtduke,J., Yamamoto,K.R., Gustafsson,J.A., and Kaptein,R. (1990). Solution structure of the glucocorticoid receptor DNA-binding domain. Science *249*, 157-160.

Harrison,R.W., Balasubramanian,K., Yeakley,J., Fant,M., Svec,F., and Fairfield,S. (1979). Heterogeneity of AtT-20 cell glucocorticoid binding sites: evidence for a membrane receptor. Advances in Experimental Medicine and Biology *117*, 423-440.

Haynes,L.E., Griffiths,M.R., Hyde,R.E., Barber,D.J., and Mitchell,I.J. (2001). Dexamethasone induces limited apoptosis and extensive sublethal damage to specific subregions of the striatum and hippocampus: implications for mood disorders. Neuroscience *104*, 57-69.

Heck,S., Kullmann,M., Gast,A., Ponta,H., Rahmsdorf,H.J., Herrlich,P., and Cato,A.C. (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. Embo Journal *13*, 4087-4095.

Helmberg,A., Auphan,N., Caelles,C., and Karin,M. (1995). Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. Embo Journal *14*, 452-460.

Henriksson,A., Almlof,T., Ford,J., McEwan,I.J., Gustafsson,J.A., and Wright,A.P. (1997). Role of the Ada adaptor complex in gene activation by the glucocorticoid receptor. Molecular and Cellular Biology *17*, 3065-3073.

Henry,C., Kabbaj,M., Simon,H., Le Moal, and Maccari,S. (1994). Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. Journal of Neuroendocrinology *6*, 341-345.

Herman,J.G., Graff,J.R., Myohanen,S., Nelkin,B.D., and Baylin,S.B. (1996). Methylation-specific PCR: a novel PCR assay for methylation status of CPG islands. Proceedings of the National Academy of Sciences of the United States of America *93*, 9821-9826.

Herman,J.P. and Cullinan,W.E. (1997). Neurocircuitry of stress: central control of the hypothalamo-pituitary-adrenocortical axis. Trends in Neurosciences *20*, 78-84.

Herman,J.P., Patel,P.D., Akil,H., and Watson,S.J. (1989a). Localization and regulation of glucocorticoid and mineralocorticoid receptor messenger-RNAs in the hippocampal- formation of the rat. Molecular Endocrinology *3*, 1886-1894.

Herman,J.P., Schafer,M.K., Thompson,R.C., and Watson,S.J. (1992). Rapid regulation of corticotropin-releasing hormone gene transcription in vivo. Molecular Endocrinology *6*, 1061-1069.

Herman,J.P., Schafer,M.K., Young,E.A., Thompson,R., Douglass,J., Akil,H., and Watson,S.J. (1989b). Evidence for hippocampal regulation of neuroendocrine neurons of the hypothalamo-pituitary-adrenocortical axis. Journal of Neuroscience *9*, 3072-3082.

Herman,J.P. and Spencer,R. (1998). Regulation of hippocampal glucocorticoid receptor gene transcription and protein expression in vivo. Journal of Neuroscience *18*, 7462-7473.

Hermann,G., Tovar,C.A., Beck,F.M., Allen,C., and Sheridan,J.F. (1993). Restraint stress differentially affects the pathogenesis of an experimental influenza viral infection in three inbred strains of mice. Journal of Neuroimmunology *47*, 83-94.

Hery,M., Semont,A., Fache,M.P., Faudon,M., and Hery,F. (2000). The effects of serotonin on glucocorticoid receptor binding in rat raphe nuclei and hippocampal cells in culture. Journal of Neurochemistry *74*, 406-413.

Hinz,B. and Hirschelmann,R. (2000). Rapid non-genomic feedback effects of glucocorticoids on CRF-induced ACTH secretion in rats. Pharmaceutical Research *17*, 1273-1277.

Hittelman,A.B., Burakov,D., Iniguez-Lluhi,J.A., Freedman,L.P., and Garabedian,M.J. (1999). Differential regulation of glucocorticoid receptor transcriptional activation via AF-1-associated proteins. Embo Journal *18*, 5380-5388.

Hollenberg,S.M., Giguere,V., Segui,P., and Evans,R.M. (1987). Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell *49*, 39-46.

Hollenberg,S.M., Weinberger,C., Ong,E.S., Cerelli,G., Oro,A., Lebo,R., Thompson,E.B., Rosenfeld,M.G., and Evans,R.M. (1985). Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature *318*, 635-641.

Holmes,M.C., French,K.L., and Seckl,J.R. (1995a). Modulation of serotonin and corticosteroid receptor gene expression in the rat hippocampus with circadian rhythm and stress. Brain Research.Molecular Brain Research *28*, 186-192.

Holmes,M.C., Kotelevtsev,Y., Mullins,J.J., and Seckl,J.R. (2001). Phenotypic analysis of mice bearing targeted deletions of 11beta-hydroxysteroid dehydrogenases 1 and 2 genes. Molecular and Cellular Endocrinology *171*, 15-20.

Holmes,M.C., Yau,J.L., French,K.L., and Seckl,J.R. (1995b). The effect of adrenalectomy on 5-hydroxytryptamine and corticosteroid receptor subtype messenger RNA expression in rat hippocampus. Neuroscience *64*, 327-337.

Htun,H., Barsony,J., Renyi,I., Gould,D.L., and Hager,G.L. (1996). Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. Proceedings of the National Academy of Sciences of the United States of America *93*, 4845-4850.

Hu,Z., Yuri,K., Ozawa,H., Lu,H., and Kawata,M. (1997). The in vivo time course for elimination of adrenalectomy-induced apoptotic profiles from the granule cell layer of the rat hippocampus. Journal of Neuroscience *17*, 3981-3989.

Hua,S.Y. and Chen,Y.Z. (1989). Membrane receptor-mediated electrophysiological effects of glucocorticoid on mammalian neurons. Endocrinology *124*, 687-691.

Hunter,T. and Karin,M. (1992). The regulation of transcription by phosphorylation. Cell *70*, 375-387.

Iwasaki,Y., Aoki,Y., Katahira,M., Oiso,Y., and Saito,H. (1997). Non-genomic mechanisms of glucocorticoid inhibition of adrenocorticotropin secretion: possible involvement of GTP-binding protein. Biochemical and Biophysical Research Communications *235*, 295-299.

Iwata,M., Hanaoka,S., and Sato,K. (1991). Rescue of thymocytes and T cell hybridomas from glucocorticoid-induced apoptosis by stimulation via the T cell receptor/CD3 complex: a possible in vitro model for positive selection of the T cell repertoire. European Journal of Immunology *21*, 643-648.

Iynedjian,P.B. (1993). Mammalian glucokinase and its gene. Biochemical Journal *293 (Pt 1)*, 1-13.

Jacobs,S.M., Gorse,K.M., and Westin,E.H. (1994). Identification of a second promoter in the human c-myb proto-oncogene. Oncogene *9*, 227-235.

Jacobson,L. and Sapolsky,R. (1991). The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocrine Reviews *12*, 118-134.

Jaenisch,R. and Jahner,D. (1984). Methylation, expression and chromosomal position of genes in mammals. Biochimica et Biophysica Acta *782*, 1-9.

Jamieson,C.A. and Yamamoto,K.R. (2000). Crosstalk pathway for inhibition of glucocorticoid-induced apoptosis by T cell receptor signaling. Proceedings of the National Academy of Sciences of the United States of America *97*, 7319-7324.

Jarrard,D.F., Kinoshita,H., Shi,Y., Sandefur,C., Hoff,D., Meisner,L.F., Chang,C.S., Herman,J.G., Isaacs,W.B., and Nassif,N. (1998). Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. Cancer Research *58*, 5310-5314.

Javahery,R., Khachi,A., Lo,K., Zenziegregory,B., and Smale,S.T. (1994). DNA-sequence requirements for transcriptional initiator activity in mammalian-cells. Molecular and Cellular Biology *14*, 116-127.

Jenkins,B.D., Pullen,C.B., and Darimont,B.D. (2001). Novel glucocorticoid receptor coactivator effector mechanisms. Trends in Endocrinology and Metabolism *12*, 122-126.

Jenson,M., Kilroy,G., York,D.A., and Braymer,D. (1996). Abnormal regulation of hepatic glucocorticoid receptor mRNA and receptor protein distribution in the obese Zucker rat. Obesity Research *4*, 133-143.

Jiang,W., Uht,R., and Bohn,M.C. (1989). Regulation of phenylethanolamine N-methyltransferase (PNMT) mRNA in the rat adrenal medulla by corticosterone. International Journal of Developmental Neuroscience *7* , 513-520.

Joels,M. and De,K. (1989). Effects of glucocorticoids and norepinephrine on the excitability in the hippocampus. Science *245* , 1502-1505.

Jondal,M., Okret,S., and McConkey,D. (1993). Killing of immature CD4+ CD8+ thymocytes in vivo by anti-CD3 or 5'-(N-ethyl)-carboxamide adenosine is blocked by glucocorticoid receptor antagonist RU-486. European Journal of Immunology *23*, 1246-1250.

Jones,C.G. and Titheradge,M.A. (1996). Measurement of metabolic fluxes through pyruvate kinase, phosphoenolpyruvate carboxykinase, pyruvate dehydrogenase, and pyruvate carboxylase in hepatocytes of different acinar origin. Archives of Biochemistry and Biophysics *326*, 202-206.

Jones,P.A. (1999). The DNA methylation paradox. Trends in Genetics *15*, 34-37.

Joseph,R. (1998). Traumatic amnesia, repression, and hippocampus injury due to emotional stress, corticosteroids and enkephalins. Child Psychiatry & Human Development *29*, 169-185.

Jungermann,K. and Kietzmann,T. (1996). Zonation of parenchymal and nonparenchymal metabolism in liver. Annual Review of Nutrition *16*, 179-203.

Kalinyak,J.E., Dorin,R.I., Hoffman,A.R., and Perlman,A.J. (1987). Tissue-specific regulation of glucocorticoid receptor messenger-RNA by dexamethasone. Journal of Biological Chemistry *262*, 10441-10444.

Karanth,S., Linthorst,A.C., Stalla,G.K., Barden,N., Holsboer,F., and Reul,J.M. (1997). Hypothalamic-pituitary-adrenocortical axis changes in a transgenic mouse with impaired glucocorticoid receptor function. Endocrinology *138*, 3476-3485.

Karin,M. (1998). New twists in gene regulation by glucocorticoid receptor: Is DNA binding dispensable? Cell *93*, 487-490.

Karin,M. and Chang,L. (2001). AP-1--glucocorticoid receptor crosstalk taken to a higher level. Journal of Endocrinology *169*, 447-451.

Karl,M., Lamberts,S.W., Koper,J.W., Katz,D.A., Huizenga,N.E., Kino,T., Haddad,B.R., Hughes,M.R., and Chrousos,G.P. (1996). Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proceedings of the Association of American Physicians *108*, 296-307.

Karst,H., Karten,Y.J., Reichardt,H.M., De,K., Schutz,G., and Joels,M. (2000). Corticosteroid actions in hippocampus require DNA binding of glucocorticoid receptor homodimers. Nature Neuroscience *3*, 977-978.

Karten,Y.J., Nair,S.M., van Essen,L., Sibug,R., and Joels,M. (1999). Long-term exposure to high corticosterone levels attenuates serotonin responses in rat hippocampal CA1 neurons. Proceedings of the National Academy of Sciences of the United States of America *96*, 13456-13461.

Kastner,P., Krust,A., Turcotte,B., Stropp,U., Tora,L., Gronemeyer,H., and Chambon,P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. Embo Journal *9*, 1603-1614.

Kel,O.V., Romaschenko,A.G., Kel,A.E., Wingender,E., and Kolchanov,N.A. (1995). A compilation of composite regulatory elements affecting gene- transcription in vertebrates. Nucleic Acids Research *23*, 4097-4103.

Kellendonk,C., Eiden,S., Kretz,O., Schutz,G., Schmidt,I., Tronche,F., and Simon,E. (2002). Inactivation of the GR in the nervous system affects energy accumulation. Endocrinology *143*, 2333-2340.

Keller-Wood,M. and Dallman,M.F. (1984). Corticosteroid inhibition of ACTH secretion. Endocrine Reviews *5*, 1-24.

Kerr,J.E., Beck,S.G., and Handa,R.J. (1996). Androgens modulate glucocorticoid receptor mRNA, but not mineralocorticoid receptor mRNA levels, in the rat hippocampus. Journal of Neuroendocrinology *8*, 439-447.

Keyser,D.O. and Pellmar,T.C. (1994). Synaptic transmission in the hippocampus: critical role for glial cells. Glia *10*, 237-243.

Kim,C.K., Yu,W., Edin,G., Ellis,L., Osborn,J.A., and Weinberg,J. (1999). Chronic intermittent stress does not differentially alter brain corticosteroid receptor densities in rats prenatally exposed to ethanol. Psychoneuroendocrinology *24*, 585-611.

King,L.B., Vacchio,M.S., Dixon,K., Hunziker,R., Margulies,D.H., and Ashwell,J.D. (1995). A targeted glucocorticoid receptor antisense transgene increases thymocyte apoptosis and alters thymocyte development. Immunity *3*, 647-656.

Kitraki,E., Karandrea,D., and Kittas,C. (1999). Long-lasting effects of stress on glucocorticoid receptor gene expression in the rat brain. Neuroendocrinology *69*, 331-338.

Kniss,D.A. and Burry,R.W. (1985). Glucocorticoid hormones inhibit DNA synthesis in glial cells cultured in chemically defined medium. Experimental Cell Research *161*, 29-40.

Kos,M., Brien,S., Flouriot,G., and Gannon,F. (2000). Tissue-specific expression of multiple mRNA variants of the mouse estrogen receptor alpha gene. Febs Letters *477*, 15-20.

Kotelevtsev,Y., Brown,R.W., Fleming,S., Kenyon,C., Edwards,C.R.W., Seckl,J.R., and Mullins,A.J. (1999). Hypertension in mice lacking 11 beta-hydroxysteroid dehydrogenase type 2. Journal of Clinical Investigation *103*, 683-689.

Kotelevtsev,Y., Holmes,M.C., Burchell,A., Houston,P.M., Schmoll,D., Jamieson,P., Best,R., Brown,R., Edwards,C.R.W., Seckl,J.R., and Mullins,J.J. (1997). 11 beta-hydroxysteroid dehydrogenase type 1 knockout mice show attenuated glucocorticoid-inducible responses and resist hyperglycemia on obesity or stress. Proceedings of the National Academy of Sciences of the United States of America *94*, 14924-14929.

Kovacs,G.L., Telegdy,G., and Lissak,K. (1977). Dose-dependent action of corticosteroids on brain serotonin content and passive avoidance behavior. Hormones and Behavior *8*, 155-165.

Kovacs,K.J. and Makara,G.B. (1988). Corticosterone and dexamethasone act at different brain sites to inhibit adrenalectomy-induced adrenocorticotropin hypersecretion. Brain Research *474*, 205-210.

Kozak,M. (1987). An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Research *15*, 8125-8148.

Kozak,M. (1991). An analysis of vertebrate mRNA sequences: intimations of translational control. The Journal of Cell Biology *115*, 887-903.

Kress,C., Thomassin,H., and Grange,T. (2001). Local DNA demethylation in vertebrates: how could it be performed and targeted? Febs Letters *494*, 135-140.

Kretz,O., Reichardt,H.M., Schutz,G., and Bock,R. (1999). Corticotropin-releasing hormone expression is the major target for glucocorticoid feedback-control at the hypothalamic level. Brain Research *818*, 488-491.

Krozowski,Z., Li,K.X., Koyama,K., Smith,R.E., Obeyesekere,V.R., Stein,O., Sasano,H., Coulter,C., Cole,T., and Sheppard,K.E. (1999). The type I and type II 11beta-hydroxysteroid dehydrogenase enzymes. Journal of Steroid Biochemistry and Molecular Biology *69*, 391-401.

Kumar,R. and Thompson,E.B. (1999). The structure of the nuclear hormone receptors. Steroids *64*, 310-319.

Kumar,S., Cole,R., Chiappelli,F., and de,V. (1989). Differential regulation of oligodendrocyte markers by glucocorticoids: post-transcriptional regulation of both proteolipid protein and myelin basic protein and transcriptional regulation of glycerol phosphate dehydrogenase. Proceedings of the National Academy of Sciences of the United States of America *86*, 6807-6811.

Kwak,S.P., Patel,P.D., Thompson,R.C., Akil,H., and Watson,S.J. (1993). 5'-Heterogeneity of the mineralocorticoid receptor messenger- ribonucleic-acid - differential expression and regulation of splice variants within the rat hippocampus. Endocrinology *133*, 2344-2350.

Landfield,P.W., Baskin,R.K., and Pitler,T.A. (1981a). Brain aging correlates: retardation by hormonal-pharmacological treatments. Science *214*, 581-584.

Landfield,P.W., Braun,L.D., Pitler,T.A., Lindsey,J.D., and Lynch,G. (1981b). Hippocampal aging in rats: a morphometric study of multiple variables in semithin sections. Neurobiology of Aging *2*, 265-275.

Lathe,R. (2001). Hormones and the hippocampus. Journal of Endocrinology *169*, 205-231.

Lechner,O., Wiegers,G.J., Oliveira-Dos-Santos,A.J., Dietrich,H., Recheis,H., Waterman,M., Boyd,R., and Wick,G. (2000). Glucocorticoid production in the murine thymus. European Journal of Immunology *30*, 337-346.

Leclerc,S., Xie,B.X., Roy,R., and Govindan,M.V. (1991). Purification of a human glucocorticoid receptor gene promoter- binding protein - production of polyclonal antibodies against the purified factor. Journal of Biological Chemistry *266*, 8711-8719.

LeGoascogne,C., Robel,P., Gouezou,M., Sananes,N., Baulieu,E.E., and Waterman,M. (1987). Neurosteroids: cytochrome P-450scc in rat brain. Science *237*, 1212-1215.

Lemaigre,F.P. and Rousseau,G.G. (1994). Transcriptional control of genes that regulate glycolysis and gluconeogenesis in adult liver. Biochemical Journal *303*, 1-14.

Levitt,N.S., Lindsay,R.S., Holmes,M.C., and Seckl,J.R. (1996). Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology *64*, 412-418.

Liu,D., Diorio,J., Tannenbaum,B., Caldji,C., Francis,D., Freedman,A., Sharma,S., Pearson,D., Plotsky,P.M., and Meaney,M.J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science *277*, 1659-1662.

Livingstone,D.E., Jones,G.C., Smith,K., Jamieson,P.M., Andrew,R., Kenyon,C.J., and Walker,B.R. (2000). Understanding the role of glucocorticoids in obesity: tissue-specific alterations of corticosterone metabolism in obese Zucker rats. Endocrinology *141*, 560-563.

Low,S.C., Moisan,M.P., Noble,J.M., Edwards,C.R.W., and Seckl,J.R. (1994). Glucocorticoids regulate hippocampal 11-beta-hydroxysteroid dehydrogenase-activity and gene-expression in-vivo in the rat. Journal of Neuroendocrinology *6*, 285-290.

Lowy,M.T. (1989). Quantification of type I and II adrenal steroid receptors in neuronal, lymphoid and pituitary tissues. Brain Research *503*, 191-197.

Luisi,B.F., Xu,W.X., Otwinowski,Z., Freedman,L.P., Yamamoto,K.R., and Sigler,P.B. (1991). Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature *352*, 497-505.

Macleod,D., Charlton,J., Mullins,J., and Bird,A.P. (1994). Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. Genes Dev *8*, 2282-2292.

Magarinos,A.M. and McEwen,B.S. (1995). Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. Neuroscience *69*, 89-98.

Maldonado,E. and Reinberg,D. (1995). News on initiation and elongation of transcription by RNA- polymerase-II. Current Opinion in Cell Biology *7*, 352-361.

Malkoski,S.P., Handanos,C.M., and Dorin,R.I. (1997). Localization of a negative glucocorticoid response element of the human corticotropin releasing hormone gene. Molecular and Cellular Endocrinology *127*, 189-199.

Mallampalli,R.K., Walter,M.E., Peterson,M.W., and Hunninghake,G.W. (1994). Betamethasone activation of CTP:cholinephosphate cytidylyltransferase in vivo is lipid dependent. American Journal of Respiratory Cell and Molecular Biology *10*, 48-57.

Marcu,K.B., Bossone,S.A., and Patel,A.J. (1992). myc function and regulation. Annual Review of Biochemistry *61*, 809-860.

Marker,A.J., Colosia,A.D., Tauler,A., Solomon,D.H., Cayre,Y., Lange,A.J., el,M., and Pilkis,S.J. (1989). Glucocorticoid regulation of hepatic 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase gene expression. Journal of Biological Chemistry *264*, 7000-7004.

Marmorstein,R. and Roth,S.Y. (2001). Histone acetyltransferases: function, structure, and catalysis. Current Opinion in Genetics & Development *11*, 155-161.

Massaad,C., Houard,N., Lombes,M., and Barouki,R. (1999). Modulation of human mineralocorticoid receptor function by protein kinase A. Molecular Endocrinology *13*, 57-65.

McCance,D.R., Pettitt,D.J., Hanson,R.L., Jacobsson,L.T., Knowler,W.C., and Bennett,P.H. (1994). Birth weight and non-insulin dependent diabetes: thrifty genotype, thrifty phenotype, or surviving small baby genotype? British Medical Journal *308*, 942-945.

McCormick,J.A., Lyons,V., Jacobson,M.D., Noble,J., Diorio,J., Nyirenda,M., Weaver,S., Ester,W., Yau,J.L.W., Meaney,M.J., Seckl,J.R., and Chapman,K.E. (2000). 5 '-Heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. Molecular Endocrinology *14*, 506-517.

McCormick,S.M. and Mendelson,C.R. (1994). Human SP-A1 and SP-A2 genes are differentially regulated during development and by cAMP and glucocorticoids. American Journal of Physiology *266*, L367-L374.

McEwan,I.J., Wright,A.P., Dahlman,W., Carlstedt,D., and Gustafsson,J.A. (1993). Direct interaction of the tau 1 transactivation domain of the human glucocorticoid receptor with the basal transcriptional machinery. Molecular and Cellular Biology *13*, 399-407.

McEwen,B.S. (1999). Stress and hippocampal plasticity. Annual Review of Neuroscience *22*, 105-122.

McEwen,B.S., Biron,C.A., Brunson,K.W., Bulloch,K., Chambers,W.H., Dhabhar,F.S., Goldfarb,R.H., Kitson,R.P., Miller,A.H., Spencer,R.L., and Weiss,J.M. (1997). The role of adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Research.Brain Research Reviews *23*, 79-133.

McEwen,B.S., deKloet,E.R., and Rostene,W. (1986). Adrenal-steroid receptors and actions in the nervous-system. Physiological Reviews *66*, 1121-1188.

McEwen,B.S. and Sapolsky,R.M. (1995). Stress and cognitive function. Current Opinion in Neurobiology *5*, 205-216.

McNally,J.G., Muller,W.G., Walker,D., Wolford,R., and Hager,G.L. (2000). The glucocorticoid receptor: rapid exchange with regulatory sites in living cells. Science *287*, 1262-1265.

McQuade,R. and Young,A.H. (2000). Future therapeutic targets in mood disorders: the glucocorticoid receptor. British Journal of Psychiatry *177*, 390-395.

Meaney,M.J. and Aitken,D.H. (1985). The effects of early postnatal handling on hippocampal glucocorticoid receptor concentrations: temporal parameters. Brain Research *354*, 301-304.

Meaney,M.J., Aitken,D.H., Bodnoff,S.R., Iny,L.J., Tatarewicz,J.E., and Sapolsky,R.M. (1985). Early postnatal handling alters glucocorticoid receptor concentrations in selected brain regions. Behavioral Neuroscience *99*, 765-770.

Meaney,M.J., Aitken,D.H., and Sapolsky,R.M. (1987). Thyroid hormones influence the development of hippocampal glucocorticoid receptors in the rat: a mechanism for the effects of postnatal handling on the development of the adrenocortical stress response. Neuroendocrinology *45*, 278-283.

Meaney,M.J., Aitken,D.H., Vanberkel,C., Bhatnagar,S., and Sapolsky,R.M. (1988). Effect of neonatal handling on age-related impairments associated with the hippocampus. Science *239*, 766-768.

Meaney,M.J., Aitken,D.H., Viau,V., Sharma,S., and Sarrieau,A. (1989). Neonatal handling alters adrenocortical negative feedback sensitivity and hippocampal type II glucocorticoid receptor binding in the rat. Neuroendocrinology *50*, 597-604.

Meaney,M.J., Diorio,J., Francis,D., Weaver,S., Yau,J., Chapman,K., and Seckl,J.R. (2000). Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: The effects of thyroid hormones and serotonin. Journal of Neuroscience *20*, 3926-3935.

Meaney,M.J., Diorio,J., Francis,D., Widdowson,J., LaPlante,P., Caldji,C., Sharma,S., Seckl,J.R., and Plotsky,P.M. (1996). Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. Developmental Neuroscience *18*, 49-72.

Meerlo,P., Horvath,K.M., Nagy,G.M., Bohus,B., and Koolhaas,J.M. (1999). The influence of postnatal handling on adult neuroendocrine and behavioural stress reactivity. Journal of Neuroendocrinology *11*, 925-933.

Meijer,O.C., Van Oosten,R.V., and de Kloet,E.R. (1997). Elevated basal trough levels of corticosterone suppress hippocampal 5-hydroxytryptamine(1A) receptor expression in adrenally intact rats: implication for the pathogenesis of depression. Neuroscience *80*, 419-426.

Mellon,S.H. and Deschepper,C.F. (1993). Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Research *629*, 283-292.

Meltzer,H.Y. (1990). Role of serotonin in depression. Annals of the New York Academy of Sciences *600*, 486-499.

Mendelson,C.R. (2000). Role of transcription factors in fetal lung development and surfactant protein gene expression. Annual Review of Physiology *62*, 875-915.

Michelsohn,A.M. and Anderson,D.J. (1992). Changes in competence determine the timing of 2 sequential glucocorticoid effects on sympathoadrenal progenitors. Neuron *8*, 589-604.

Miesfeld,R., Rusconi,S., Godowski,P.J., Maler,B.A., Okret,S., Wikstrom,A.C., Gustafsson,J.A., and Yamamoto,K.R. (1986). Genetic complementation of a glucocorticoid receptor deficiency by expression of cloned receptor cDNA. Cell *46*, 389-399.

Miller,A.H., Spencer,R.L., Pearce,B.D., Pisell,T.L., Azrieli,Y., Tanapat,P., Moday,H., Rhee,R., and McEwen,B.S. (1998). Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. Cellular Immunology *186*, 45-54.

Miller,A.H., Spencer,R.L., Stein,M., and McEwen,B.S. (1990). Adrenal steroid receptor binding in spleen and thymus after stress or dexamethasone. American Journal of Physiology *259*, E405-E412.

Minderop,R.H., Hoeppner,W., and Seitz,H.J. (1987). Regulation of hepatic glucokinase gene expression. Role of carbohydrates, and glucocorticoid and thyroid hormones. European Journal of Biochemistry *164*, 181-187.

Mitchell,J.B., Betito,K., Rowe,W., Boksa,P., and Meaney,M.J. (1992). Serotonergic regulation of type-II corticosteroid receptor- binding in hippocampal cell-cultures - evidence for the importance of serotonin-induced changes in cAMP levels. Neuroscience *48*, 631-639.

Mitchell,J.B., Iny,L.J., and Meaney,M.J. (1990a). The role of serotonin in the development and environmental- regulation of type-II corticosteroid receptor-binding in rat hippocampus. Developmental Brain Research *55*, 231-235.

Mitchell,J.B., Rowe,W., Boksa,P., and Meaney,M.J. (1990b). Serotonin regulates type-II corticosteroid receptor-binding in hippocampal cell-cultures. Journal of Neuroscience *10*, 1745-1752.

Mizoguchi,K., Yuzurihara,M., Ishige,A., Sasaki,H., Chui,D.H., and Tabira,T. (2001). Chronic stress differentially regulates glucocorticoid negative feedback response in rats. Psychoneuroendocrinology *26*, 443-459.

Molina,C.A., Foulkes,N.S., Lalli,E., and Sassone,C. (1993). Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell *75*, 875-886.

Morale,M.C., Batticane,N., Gallo,F., Barden,N., and Marchetti,B. (1995). Disruption of hypothalamic-pituitary-adrenocortical system in transgenic mice expressing type II glucocorticoid receptor antisense ribonucleic acid permanently impairs T cell function: effects on T cell trafficking and T cell responsiveness during postnatal development. Endocrinology *136*, 3949-3960.

Morita,K., Ishimura,K., Tsuruo,Y., and Wong,D.L. (1999). Dexamethasone enhances serum deprivation-induced necrotic death of rat C6 glioma cells through activation of glucocorticoid receptors. Brain Research *816*, 309-316.

Mu,Y.M., Takayanagi,R., Imasaki,K., Ohe,K., Ikuyama,S., Yanase,T., and Nawata,H. (1998). Low level of glucocorticoid receptor messenger ribonucleic acid in pituitary adenomas manifesting Cushing's disease with resistance to a high dose-dexamethasone suppression test. Clinical Endocrinology (Oxford) *49*, 301-306.

Muglia,L.J., Bae,D.S., Brown,T.T., Vogt,S.K., Alvarez,J.G., Sunday,M.E., and Majzoub,J.A. (1999). Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. American Journal of Respiratory Cell and Molecular Biology *20*, 181-188.

Munck,A., Crabtree,G.R., and Smith,K.A. (1979). Glucocorticoid receptors and actions in rat thymocytes and immunologically stimulated human peripheral lymphocytes. Monographs On Endocrinology *12*, 341-355.

Munck,A., Guyre,P.M., and Holbrook,N.J. (1984). Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocrine Reviews *5*, 25-44.

Nagaya,M., Arai,M., and Widmaier,E.P. (1995). Ontogeny of immunoreactive and bioactive microsomal steroidogenic enzymes during adrenocortical development in rats. Molecular and Cellular Endocrinology *114*, 27-34.

Nagpal,S., Friant,S., Nakshatri,H., and Chambon,P. (1993). RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization in vivo. Embo Journal *12*, 2349-2360.

Nebes,V.L. and Morris,S.M. (1988). Regulation of messenger ribonucleic acid levels for five urea cycle enzymes in cultured rat hepatocytes. Requirements for cyclic adenosine monophosphate, glucocorticoids, and ongoing protein synthesis. Molecular Endocrinology *2*, 444-451.

Nemeroff,C.B., Widerlov,E., Bissette,G., Walleus,H., Karlsson,I., Eklund,K., Kilts,C.D., Loosen,P.T., and Vale,W. (1984). Elevated concentrations of CSF corticotropin-releasing factor-like immunoreactivity in depressed patients. Science *226*, 1342-1344.

Neumaier,J.F., Sexton,T.J., Hamblin,M.W., and Beck,S.G. (2000). Corticosteroids regulate 5-HT(1A) but not 5-HT(1B) receptor mRNA in rat hippocampus. Brain Research.Molecular Brain Research *82*, 65-73.

Newell-Price,J., Clark,A.J., and King,P. (2000). DNA methylation and silencing of gene expression. Trends in Endocrinology and Metabolism *11*, 142-148.

Niimi,S., Yamaguchi,T., and Hayakawa,T. (1997). Regulation of glucocorticoid receptor by the tyrosine kinase inhibitor herbimycin A in the cytosolic fraction of primary cultured rat hepatocytes. Journal of Steroid Biochemistry and Molecular Biology *61*, 65-71.

Nishio,H., Takeshima,Y., Narita,N., Yanagawa,H., Suzuki,Y., Ishikawa,Y., Ishikawa,Y., Minami,R., Nakamura,H., and Matsuo,M. (1994). Identification of a novel first exon in the human dystrophin gene and of a new promoter located more than 500 kb upstream of the nearest known promoter. The Journal of Clinical Investigation *94*, 1037-1042.

Nobukuni,Y., Smith,C.L., Hager,G.L., and Detera-Wadleigh,S.D. (1995). Characterisation of the human glucocorticoid receptor promoter. Biochemistry *34*, 8207-8214.

Nunez,B.S. and Vedeckis,W.V. (2002). Characterization of promoter 1B in the human glucocorticoid receptor gene. Molecular and Cellular Endocrinology *189*, 191-199.

Nussdorfer,G.G., Bahcelioglu,M., Neri,G., and Malendowicz,L.K. (2000). Secretin, glucagon, gastric inhibitory polypeptide, parathyroid hormone, and related peptides in the regulation of the hypothalamus- pituitary-adrenal axis. Peptides *21*, 309-324.

Nyirenda,M.J., Lindsay,R.S., Kenyon,C.J., Burchell,A., and Seckl,J.R. (1998). Glucocorticoid exposure in late gestation permanently programs rat hepatic phosphoenolpyruvate carboxykinase and glucocorticoid receptor expression and causes glucose intolerance in adult offspring. Journal of Clinical Investigation *101*, 2174-2181.

O'Donnell,D., Francis,D., Weaver,S., and Meaney,M.J. (1995). Effects of adrenalectomy and corticosterone replacement on glucocorticoid receptor levels in rat brain tissue: a comparison between western blotting and receptor binding assays. Brain Research *687*, 133-142.

O'Donnell,D., Larocque,S., Seckl,J.R., and Meaney,M.J. (1994). Postnatal handling alters glucocorticoid, but not mineralocorticoid messenger-RNA expression in the hippocampus of adult-rats. Molecular Brain Research *26*, 242-248.

Oakley,R.H., Jewell,C.M., Yudt,M.R., Bofetiado,D.M., and Cidlowski,J.A. (1999). The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. Journal of Biological Chemistry *274*, 27857-27866.

Oakley,R.H., Sar,M., and Cidlowski,J.A. (1996). The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. Journal of Biological Chemistry *271*, 9550-9559.

Oitzl,M.S., de Kloet,E.R., Joels,M., Schmid,W., and Cole,T.J. (1997). Spatial learning deficits in mice with a targeted glucocorticoid receptor gene disruption. European Journal of Neuroscience *9*, 2284-2296.

Oitzl,M.S. and DeKloet (1992). Selective corticosteroid antagonists modulate specific aspects of spatial orientation learning. Behavioral Neuroscience *106*, 62-71.

Oitzl,M.S., van Haarst,A.D., Sutanto,W., and de Kloet,E.R. (1995). Corticosterone, brain mineralocorticoid receptors (MRs) and the activity of the hypothalamic-pituitary-adrenal (HPA) axis: the Lewis rat as an example of increased central MR capacity and a hyporesponsive HPA axis. Psychoneuroendocrinology *20*, 655-675.

Okugawa,G., Omori,K., Suzukawa,J., Fujiseki,Y., Kinoshita,T., and Inagaki,C. (1999). Long-term treatment with antidepressants increases glucocorticoid receptor binding and gene expression in cultured rat hippocampal neurones. Journal of Neuroendocrinology *11*, 887-895.

Orchinik,M., Murray,T.F., and Moore,F.L. (1991). A corticosteroid receptor in neuronal membranes. Science *252*, 1848-1851.

Orth,D.N. (1998). The Adrenal Cortex. In Williams Textbook of Endocrinology, (Philadelphia: W.B.Saunders), pp. 517-664.

Osterlund,M.K., Grandien,K., Keller,E., and Hurd,Y.L. (2000). The human brain has distinct regional expression patterns of estrogen receptor alpha mRNA isoforms derived from alternative promoters. Journal of Neurochemistry *75*, 1390-1397.

Otto,C., Reichardt,H.M., and Schutz,G. (1997). Absence of glucocorticoid receptor-beta in mice. Journal of Biological Chemistry *272*, 26665-26668.

Pardridge,W.M. (1987). Plasma protein-mediated transport of steroid and thyroid hormones. American Journal of Physiology *252* , E157-E164.

Pariante,C.M. and Miller,A.H. (2001). Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment. Biological Psychiatry *49*, 391-404.

Pariante,C.M., Pearce,B.D., Pisell,T.L., Owens,M.J., and Miller,A.H. (1997). Steroid-independent translocation of the glucocorticoid receptor by the antidepressant desipramine. Molecular Pharmacology *52*, 571-581.

Parker,K.L. and Schimmer,B.P. (1993). Transcriptional regulation of the adrenal steroidogenic enzymes. Trends in Endocrinology and Metabolism *4*, 46-50.

Paskitti,M.E., McCreary,B.J., and Herman,J.P. (2000). Stress regulation of adrenocorticosteroid receptor gene transcription and mRNA expression in rat hippocampus: time-course analysis. Brain Research.Molecular Brain Research *80*, 142-152.

Patacchioli,F.R., Angelucci,L., Casolini,P., Bottone,A., Borboni,P., Lauro,R., and Marlier,L.N. (1998). Corticosterone treatment differentially affects adrenocorticoid receptors expression and binding in the hippocampus and spinal cord of the rat. Journal of Molecular Neuroscience *11* , 95-103.

Pavlides,C., Watanabe,Y., and McEwen,B.S. (1993). Effects of glucocorticoids on hippocampal long-term potentiation. Hippocampus *3*, 183-192.

Pavlik,A. and Buresova,M. (1984). The neonatal cerebellum: the highest level of glucocorticoid receptors in the brain. Brain Research *314*, 13-20.

Pazirandeh,A., Xue,Y., Prestegaard,T., Jondal,M., and Okret,S. (2002). Effects of altered glucocorticoid sensitivity in the T cell lineage on thymocyte and T cell homeostasis. Faseb Journal *16*, 727-729.

Pazirandeh,A., Xue,Y.T., Rafter,I., Sjovall,J., Jondal,M., and Okret,S. (1999). Paracrine Glucocorticoid Activity Produced By Mouse Thymic Epithelial Cells. Faseb Journal *13*, 893-901.

Pearce,D. and Yamamoto,K.R. (1993). Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science *259*, 1161-1165.

Peiffer,A., Lapointe,B., and Barden,N. (1991a). Hormonal regulation of type II glucocorticoid receptor messenger ribonucleic acid in rat brain. Endocrinology *129*, 2166-2174.

Peiffer,A., Morale,M.C., Barden,N., and Marchetti,B. (1994). Modulation of glucocorticoid receptor gene-expression in the thymus by the sex steroid-hormone milieu and correlation with sexual dimorphism of immune-response. Endocrine *2*, 181-192.

Peiffer,A., Veilleux,S., and Barden,N. (1991b). Antidepressant and other centrally acting drugs regulate glucocorticoid receptor messenger RNA levels in rat brain. Psychoneuroendocrinology *16*, 505-515.

Pellerin,L. and Magistretti,P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proceedings of the National Academy of Sciences of the United States of America *91*, 10625-10629.

Pemberton,P.A., Stein,P.E., Pepys,M.B., Potter,J.M., and Carrell,R.W. (1988). Hormone binding globulins undergo serpin conformational change in inflammation. Nature *336*, 257-258.

Pepin,M.C., Beaulieu,S., and Barden,N. (1989). Antidepressants regulate glucocorticoid receptor messenger RNA concentrations in primary neuronal cultures. Brain Research.Molecular Brain Research *6*, 77-83.

Pepin,M.C., Pothier,F., and Barden,N. (1992a). Antidepressant drug-action in a transgenic mouse model of the endocrine changes seen in depression. Molecular Pharmacology *42*, 991-995.

Pepin,M.C., Pothier,F., and Barden,N. (1992b). Impaired type-II glucocorticoid-receptor function in mice bearing antisense RNA transgene. Nature *355*, 725-728.

Picard,D. and Yamamoto,K.R. (1987). Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo Journal *6*, 3333-3340.

Pierreux,C.E., Urso,B., De Meyts,P., Rousseau,G.G., and Lemaigre,F.P. (1998). Inhibition by insulin of glucocorticoid-induced gene transcription: Involvement of the ligand-binding domain of the glucocorticoid receptor and independence from the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. Molecular Endocrinology *12*, 1343-1354.

Plotsky,P.M. and Meaney,M.J. (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Brain Research.Molecular Brain Research *18*, 195-200.

Pugh,B.F. (1996). Mechanisms of transcription complex assembly. Current Opinion in Cell Biology *8*, 303-311.

Purton,J.F., Boyd,R.L., Cole,T.J., and Godfrey,D.I. (2000). Intrathymic T cell development and selection proceeds normally in the absence of glucocorticoid receptor signaling. Immunity *13*, 179-186.

Raber,J. (1998). Detrimental effects of chronic hypothalamic-pituitary-adrenal axis activation - From obesity to memory deficits. Molecular Neurobiology *18*, 1-22.

Racine,R.J., Milgram,N.W., and Hafner,S. (1983). Long-term potentiation phenomena in the rat limbic forebrain. Brain Research *260*, 217-231.

Raghow,R., Gossage,D., and Kang,A.H. (1986). Pretranslational regulation of type I collagen, fibronectin, and a 50-kilodalton noncollagenous extracellular protein by dexamethasone in rat fibroblasts. Journal of Biological Chemistry *261*, 4677-4684.

Rajan,V., Edwards,C.R.W., and Seckl,J.R. (1996). 11 beta-hydroxysteroid dehydrogenase in cultured hippocampal cells reactivates inert 11-dehydrocorticosterone, potentiating neurotoxicity. Journal of Neuroscience *16*, 65-70.

Ramdas,J., Liu,W., and Harmon,J.M. (1999). Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Research *59*, 1378-1385.

Ranelletti,F.O., Maggiano,N., Aiello,F.B., Carbone,A., Larocca,L.M., Musiani,P., and Piantelli,M. (1987). Glucocorticoid receptors and corticosensitivity of human thymocytes at discrete stages of intrathymic differentiation. Journal of Immunology *138*, 440-445.

Ratka,A., Sutanto,W., Bloemers,M., and deKloet,E.R. (1989). On the role of brain mineralocorticoid (type I) and glucocorticoid (type II) receptors in neuroendocrine regulation. Neuroendocrinology *50*, 117-123.

Reagan,L.P. and McEwen,B.S. (1997). Controversies surrounding glucocorticoid-mediated cell death in the hippocampus. Journal of Chemical Neuroanatomy *13*, 149-167.

Reichardt,H.M., Kaestner,K.H., Tuckermann,J., Kretz,O., Wessely,O., Bock,R., Gass,P., Schmid,W., Herrlich,P., Angel,P., and Schutz,G. (1998). DNA binding of the glucocorticoid receptor is not essential for survival. Cell *93*, 531-541.

Reichardt,H.M. and Schutz,G. (1998). Glucocorticoid signalling--multiple variations of a common theme. Molecular and Cellular Endocrinology *146*, 1-6.

Reichardt,H.M., Tronche,F., Bauer,A., and Schutz,G. (2000a). Molecular genetic analysis of glucocorticoid signaling using the Cre/loxP system. Biological Chemistry *381*, 961-964.

Reichardt,H.M., Umland,T., Bauer,A., Kretz,O., and Schutz,G. (2000b). Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Molecular and Cellular Biology *20*, 9009-9017.

Reik,A., Schutz,G., and Stewart,A.F. (1991). Glucocorticoids are required for establishment and maintenance of an alteration in chromatin structure: induction leads to a reversible disruption of nucleosomes over an enhancer. Embo Journal *10*, 2569-2576.

Reinisch,J.M., Simon,N.G., Karow,W.G., and Gandelman,R. (1978). Prenatal exposure to prednisone in humans and animals retards intrauterine growth. Science *202*, 436-438.

Reisman,D. and Thompson,E.A. (1995). Glucocorticoid regulation of cyclin D3 gene transcription and mRNA stability in lymphoid cells. Molecular Endocrinology *9*, 1500-1509.

Reul,J.M. and DeKloet (1985). Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology *117*, 2505-2511.

Reul,J.M., Pearce,P.T., Funder,J.W., and Krozowski,Z.S. (1989). Type I and type II corticosteroid receptor gene expression in the rat: effect of adrenalectomy and dexamethasone administration. Molecular Endocrinology *3*, 1674-1680.

Reul,J.M., van den Bosch,F., and deKloet,E.R. (1987a). Differential response of type I and type II corticosteroid receptors to changes in plasma steroid level and circadian rhythmicity. Neuroendocrinology *45*, 407-412.

Reul,J.M.H.M., van den Bosch,F., and deKloet,E.R. (1987b). Relative occupation of type-I and type-II corticosteroid receptors in rat-brain following stress and dexamethasone treatment - functional implications. Journal of Endocrinology *115*, 459-467.

Rice,J.C. and Allis,C.D. (2001). Histone methylation versus histone acetylation: new insights into epigenetic regulation. Current Opinion in Cell Biology *13*, 263-273.

Riegel,A.T., Lu,Y., Remenick,J., Wolford,R.G., Berard,D.S., and Hager,G.L. (1991). Proopiomelanocortin gene promoter elements required for constitutive and glucocorticoid-repressed transcription. Molecular Endocrinology *5*, 1973-1982.

Rigaud,G., Roux,J., Pictet,R., and Grange,T. (1991). In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell *67*, 977-986.

Robertson,K.D. and Wolffe,A.P. (2000). DNA methylation in health and disease. Nature Reviews Genetics *1*, 11-19.

Robson,A.C., Leckie,C.M., Seckl,J.R., and Holmes,M.C. (1998). 11 Beta-hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain. Brain Research.Molecular Brain Research *61*, 1-10.

Robyr,D., Wolffe,A.P., and Wahli,W. (2000). Nuclear hormone receptor coregulators in action: Diversity for shared tasks. Molecular Endocrinology *14*, 329-347.

Roozendaal,B. (2000). 1999 Curt P. Richter award. Glucocorticoids and the regulation of memory consolidation. Psychoneuroendocrinology *25*, 213-238.

Roozendaal,B. and McGaugh,J.L. (1996a). Amygdaloid nuclei lesions differentially affect glucocorticoid-induced memory enhancement in an inhibitory avoidance task. Neurobiology of Learning and Memory *65*, 1-8.

Roozendaal,B. and McGaugh,J.L. (1996b). The memory-modulatory effects of glucocorticoids depend on an intact stria terminalis. Brain Research *709*, 243-250.

Roozendaal,B. and McGaugh,J.L. (1997a). Basolateral amygdala lesions block the memory-enhancing effect of glucocorticoid administration in the dorsal hippocampus of rats. European Journal of Neuroscience *9*, 76-83.

Roozendaal,B. and McGaugh,J.L. (1997b). Glucocorticoid receptor agonist and antagonist administration into the basolateral but not central amygdala modulates memory storage. Neurobiology of Learning and Memory *67*, 176-179.

Roozendaal,B., Portillo,M., and McGaugh,J.L. (1996). Basolateral amygdala lesions block glucocorticoid-induced modulation of memory for spatial learning. Behavioral Neuroscience *110*, 1074-1083.

Roozendaal,B., Williams,C.L., and McGaugh,J.L. (1999). Glucocorticoid receptor activation in the rat nucleus of the solitary tract facilitates memory consolidation: involvement of the basolateral amygdala. European Journal of Neuroscience *11*, 1317-1323.

Rosewicz,S., McDonald,A.R., Maddux,B.A., Goldfine,I.D., Miesfeld,R.L., and Logsdon,C.D. (1988). Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. Journal of Biological Chemistry *263* , 2581-2584.

Ross,M.E., Evinger,M.J., Hyman,S.E., Carroll,J.M., Mucke,L., Comb,M., Reis,D.J., Joh,T.H., and Goodman,H.M. (1990). Identification of a functional glucocorticoid response element in the phenylethanolamine N-methyltransferase promoter using fusion genes introduced into chromaffin cells in primary culture. Journal of Neuroscience *10*, 520-530.

Sacedon,R., Vicente,A., Varas,A., Jimenez,E., Munoz,J.J., and Zapata,A.G. (1999a). Early maturation of T-cell progenitors in the absence of glucocorticoids. Blood *94*, 2819-2826.

Sacedon,R., Vicente,A., Varas,A., Morale,M.C., Barden,N., Marchetti,B., and Zapata,A.G. (1999b). Partial blockade of T-cell differentiation during ontogeny and marked alterations of the thymic microenvironment in transgenic mice with impaired glucocorticoid receptor function. Journal of Neuroimmunology *98*, 157-167.

Sadler,S.E., Bower,M.A., and Maller,J.L. (1985). Studies of a plasma membrane steroid receptor in Xenopus oocytes using the synthetic progestin RU 486. Journal of Steroid Biochemistry *22*, 419-426.

Sakai,K., Horiba,N., Sakai,Y., Tozawa,F., Demura,H., and Suda,T. (1996). Regulation of corticotropin-releasing factor receptor messenger ribonucleic acid in rat anterior pituitary. Endocrinology *137*, 1758-1763.

Sapolsky,R.M. (1985). A mechanism for glucocorticoid toxicity in the hippocampus: increased neuronal vulnerability to metabolic insults. Journal of Neuroscience *5*, 1228-1232.

Sapolsky,R.M. (2000). Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Archives of General Psychiatry *57*, 925-935.

Sapolsky,R.M., Krey,L.C., and McEwen,B.S. (1984a). Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proceedings of the National Academy of Sciences of the United States of America *81*, 6174-6177.

Sapolsky,R.M., Krey,L.C., and McEwen,B.S. (1984b). Stress down-regulates corticosterone receptors in a site- specific manner in the brain. Endocrinology *114*, 287-292.

Sapolsky,R.M., Krey,L.C., and McEwen,B.S. (1985). Prolonged glucocorticoid exposure reduces hippocampal neuron number: implications for aging. Journal of Neuroscience *5*, 1222-1227.

Sapolsky,R.M., Krey,L.C., and McEwen,B.S. (1986). The neuroendocrinology of stress and aging: the glucocorticoid cascade hypothesis. Endocrine Reviews *7*, 284-301.

Sapolsky,R.M. and McEwen,B.S. (1985). Down-regulation of neural corticosterone receptors by corticosterone and dexamethasone. Brain Research *339*, 161-165.

Sapolsky,R.M., McEwen,B.S., and Rainbow,T.C. (1983). Quantitative autoradiography of [3H]corticosterone receptors in rat brain. Brain Research *271*, 331-334.

Sapolsky,R.M. and Pulsinelli,W.A. (1985). Glucocorticoids potentiate ischemic injury to neurons: therapeutic implications. Science *229*, 1397-1400.

Sarrieau,A., Sharma,S., and Meaney,M.J. (1988). Postnatal development and environmental regulation of hippocampal glucocorticoid and mineralocorticoid receptors. Brain Research *471*, 158-162.

Sasaki,K., Cripe,T.P., Koch,S.R., Andreone,T.L., Petersen,D.D., Beale,E.G., and Granner,D.K. (1984). Multihormonal regulation of phosphoenolpyruvate carboxykinase gene transcription. The dominant role of insulin. Journal of Biological Chemistry *259*, 15242-15251.

Savoldi,G., Fenaroli,A., Ferrari,F., Rigaud,G., Albertini,A., and Di,L. (1997). The glucocorticoid receptor regulates the binding of C/EPBbeta on the alpha-1-acid glycoprotein promoter in vivo. DNA and Cell Biology *16*, 1467-1476.

Scheinman,R.I., Cogswell,P.C., Lofquist,A.K., and Baldwin,A.S. (1995). Role of transcriptional activation of I-kappa-B-alpha in mediation of immunosuppression by glucocorticoids. Science *270*, 283-286.

Schibler,U., Hagenbuchle,O., Wellauer,P.K., and Pittet,A.C. (1983). Two promoters of different strengths control the transcription of the mouse alpha-amylase gene Amy-1a in the parotid gland and the liver. Cell *33*, 501-508.

Schmoll,D., Wasner,C., Hinds,C.J., Allan,B.B., Walther,R., and Burchell,A. (1999). Identification of a cAMP response element within the glucose- 6-phosphatase hydrolytic subunit gene promoter which is involved in the transcriptional regulation by cAMP and glucocorticoids in H4IIE hepatoma cells. Biochemical Journal *338 (Pt 2)*, 457-463.

Schule,R., Muller,M., Kaltschmidt,C., and Renkawitz,R. (1988). Many transcription factors interact synergistically with steroid-receptors. Science *242*, 1418-1420.

Seckl,J.R. (1994). Glucocorticoids and small babies. Quarterly Journal of Medicine *87*, 259-262.

Seckl,J.R. (1997). 11 beta-hydroxysteroid dehydrogenase in the brain: A novel regulator of glucocorticoid action? Frontiers in Neuroendocrinology *18*, 49-99.

Seckl,J.R. and Chapman,K.E. (1997). Medical and physiological aspects of the 11beta-hydroxysteroid dehydrogenase system. European Journal of Biochemistry *249*, 361-364.

Seckl,J.R., Dickson,K.L., and Fink,G. (1990). Central 5,7-dihydroxytryptamine lesions decrease hippocampal glucocorticoid and mineralocorticoid receptor messenger- ribonucleic-acid expression. Journal of Neuroendocrinology *2*, 911-916.

Seckl,J.R. and Fink,G. (1992). Antidepressants increase glucocorticoid and mineralocorticoid receptor mRNA expression in rat hippocampus in vivo. Neuroendocrinology *55*, 621-626.

Seckl,J.R. and Olsson,T. (1995). Glucocorticoid hypersecretion and the age-impaired hippocampus - cause or effect. Journal of Endocrinology *145*, 201-211.

Seger,M.A., van,E., Kiss,J.Z., Burbach,J.P., and DeKloet,E.R. (1988). Stimulation of pro-opiomelanocortin gene expression by glucocorticoids in the denervated rat intermediate pituitary gland. Neuroendocrinology *47*, 350-357.

Semba,J., Watanabe,H., Suhara,T., and Akanuma,N. (2000). Chronic lithium chloride injection increases glucocorticoid receptor but not mineralocorticoid receptor mRNA expression in rat brain. Neuroscience Research *38*, 313-319.

Sharma,R. and Timiras,P.S. (1987). Age-dependent regulation of glucocorticoid receptors in the liver of male rats. Biochimica et Biophysica Acta *930*, 237-243.

Sheppard,K.A., Phelps,K.M., Williams,A.J., Thanos,D., Glass,C.K., Rosenfeld,M.G., Gerritsen,M.E., and Collins,T. (1998). Nuclear integration of glucocorticoid receptor and nuclear factor-kappa B signaling by CREB-binding protein and steroid receptor coactivator-1. Journal of Biological Chemistry *273*, 29291-29294.

Sheppard,K.E., Roberts,J.L., and Blum,M. (1990). Differential regulation of type II corticosteroid receptor messenger ribonucleic acid expression in the rat anterior pituitary and hippocampus. Endocrinology *127*, 431-439.

Shors,T.J., Weiss,C., and Thompson,R.F. (1992). Stress-induced facilitation of classical conditioning. Science *257*, 537-539.

Siegfried,Z., Eden,S., Mendelsohn,M., Feng,X., Tsuberi,B.Z., and Cedar,H. (1999). DNA methylation represses transcription in vivo. Nature Genetics *22*, 203-206.

Silke,J., Rother,K.I., Georgiev,O., Schaffner,W., and Matsuo,K. (1995). Complex demethylation patterns at SP1 binding-sites in F9 embryonal carcinoma-cells. Febs Letters *370*, 170-174.

Sloviter,R.S., Dean,E., and Neubort,S. (1993a). Electron microscopic analysis of adrenalectomy-induced hippocampal granule cell degeneration in the rat: apoptosis in the adult central nervous system. Journal of Comparative Neurology *330*, 337-351.

Sloviter,R.S., Sollas,A.L., Dean,E., and Neubort,S. (1993b). Adrenalectomy-induced granule cell degeneration in the rat hippocampal dentate gyrus: characterization of an in vivo model of controlled neuronal death. Journal of Comparative Neurology *330*, 324-336.

Sloviter,R.S., Valiquette,G., Abrams,G.M., Ronk,E.C., Sollas,A.L., Paul,L.A., and Neubort,S. (1989). Selective loss of hippocampal granule cells in the mature rat-brain after adrenalectomy. Science *243*, 535-538.

Smith,C.L. and Hammond,G.L. (1991). Ontogeny of corticosteroid-binding globulin biosynthesis in the rat. Endocrinology *128*, 983-988.

Smythe,J.W., Rowe,W.B., and Meaney,M.J. (1994). Neonatal Handling Alters Serotonin (5-HT) Turnover And 5-HT2 Receptor-Binding In Selected Brain-Regions - Relationship To The Handling Effect On Glucocorticoid Receptor Expression. Developmental Brain Research *80*, 183-189.

Snochowski,M., Dahlberg,E., and Gustafsson,J.A. (1980). Characterization and quantification of the androgen and glucocorticoid receptors in cytosol from rat skeletal muscle. European Journal of Biochemistry *111*, 603-616.

Sousa,R.J., Tannery,N.H., and Lafer,E.M. (1989). In situ hybridization mapping of glucocorticoid receptor messenger ribonucleic-acid in rat-brain. Molecular Endocrinology *3*, 481-494.

Spencer,R.L., Miller,A.H., Moday,H., Stein,M., and McEwen,B.S. (1993). Diurnal differences in basal and acute stress levels of type I and type II adrenal steroid receptor activation in neural and immune tissues. Endocrinology *133*, 1941-1950.

Spencer,R.L., Miller,A.H., Stein,M., and McEwen,B.S. (1991). Corticosterone regulation of type-I and type-II adrenal-steroid receptors in brain, pituitary, and immune tissue. Brain Research *549*, 236-246.

Spencer,R.L., Young,E.A., Choo,P.H., and McEwen,B.S. (1990). Adrenal steroid type I and type II receptor binding: estimates of in vivo receptor number, occupancy, and activation with varying level of steroid. Brain Research *514*, 37-48.

Sprent,J., Lo,D., Gao,E.K., and Ron,Y. (1988). T-cell selection in the thymus. Immunological Reviews *101*, 173-190.

Starr,D.B., Matsui,W., Thomas,J.R., and Yamamoto,K.R. (1996). Intracellular receptors use a common mechanism to interpret signaling information at response elements. Genes & Development *10*, 1271-1283.

Stec,I., Barden,N., Reul,J.M., and Holsboer,F. (1994). Dexamethasone nonsuppression in transgenic mice expressing antisense RNA to the glucocorticoid receptor. Journal of Psychiatric Research *28*, 1-5.

Stein,R., Orit,S., and Anderson,D.J. (1988). The induction of a neural-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, and glucocorticoid inhibitable. Developmental Biology *127*, 316-325.

Sternberg,E.M. (2001). Neuroendocrine regulation of autoimmune/inflammatory disease. Journal of Endocrinology *169*, 429-435.

Sternberg,E.M., Hill,J.M., Chrousos,G.P., Kamilaris,T., Listwak,S.J., Gold,P.W., and Wilder,R.L. (1989). Inflammatory mediator-induced hypothalamic-pituitary-adrenal axis activation is defective in streptococcal cell wall arthritis-susceptible Lewis rats. Proceedings of the National Academy of Sciences of the United States of America *86*, 2374-2378.

Sterner,D.E. and Berger,S.L. (2000). Acetylation of histones and transcription-related factors. Microbiology and Molecular Biology Reviews *64*, 435-459.

Stocklin,E., Wissler,M., Gouilleux,F., and Groner,B. (1996). Functional interactions between Stat5 and the glucocorticoid receptor. Nature *383*, 726-728.

Strahle,U., Schmid,W., and Schutz,G. (1988). Synergistic action of the glucocorticoid receptor with transcription factors. Embo Journal *7*, 3389-3395.

Strahle,U., Schmidt,A., Kelsey,G., Stewart,A.F., Cole,T.J., Schmid,W., and Schutz,G. (1992). At least 3 promoters direct expression of the mouse glucocorticoid receptor gene. Proceedings of the National Academy of Sciences of the United States of America *89*, 6731-6735.

Struhl,K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes & Development *12*, 599-606.

Suemaru,S., Darlington,D.N., Akana,S.F., Cascio,C.S., and Dallman,M.F. (1995). Ventromedial hypothalamic-lesions inhibit corticosteroid feedback-regulation of basal ACTH during the trough of the circadian-rhythm. Neuroendocrinology *61*, 453-463.

Suzuki,Y., Yamashita,R., Nakai,K., and Sugano,S. (2002). DBTSS: DataBase of human Transcriptional Start Sites and full-length cDNAs. Nucleic Acids Research *30*, 328-331.

Svec,F. (1988). Corticosterone regulates the level of hepatic glucocorticoid receptors in mice. Proceedings of the Society For Experimental Biology and Medicine *188*, 474-479.

Svec,F., Gordon,S., and Tate,D. (1989). Glucocorticoid receptor regulation: the effects of adrenalectomy, exogenous glucocorticoid, and stress on hepatic receptor number in male and female mice. Biochemical Medicine and Metabolic Biology *41*, 224-233.

Tanimura,S.M. and Watts,A.G. (2001). Corticosterone modulation of ACTH secretogogue gene expression in the paraventricular nucleus. Peptides *22*, 775-783.

Teitelman,G., Joh,T.H., Park,D., Brodsky,M., New,M., and Reis,D.J. (1982). Expression of the adrenergic phenotype in cultured fetal adrenal medullary cells: role of intrinsic and extrinsic factors. Developmental Biology *89*, 450-459.

Tenbaum,S. and Baniahmad,A. (1997). Nuclear receptors: structure, function and involvement in disease. The International Journal of Biochemistry & Cell Biology *29*, 1325-1341.

Tenhunen,J., Salminen,M., Jalanko,A., Ukkonen,S., and Ulmanen,I. (1993). Structure of the rat catechol-O-methyltransferase gene: separate promoters are used to produce mRNAs for soluble and membrane-bound forms of the enzyme. DNA Cell Biol *12*, 253-263.

Thomas,S.R., Assaf,S.Y., and Iversen,S.D. (1984). Amygdaloid complex modulates neurotransmission from the entorhinal cortex to the dentate gyrus of the rat. Brain Research *307*, 363-365.

Thomassin,H., Flavin,M., Espinas,M.L., and Grange,T. (2001). Glucocorticoid-induced DNA demethylation and gene memory during development. Embo Journal *20*, 1974-1983.

Tjian,R. and Maniatis,T. (1994). Transcriptional activation - a complex puzzle with few easy pieces. Cell *77*, 5-8.

Tonko,M., Ausserlechner,M.J., Bernhard,D., Helmberg,A., and Kofler,R. (2001). Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. Faseb Journal *15*, 693-699.

Tornello,S., Orti,E., De Nicola,T.C., Rainbow,T.C., and McEwen,B.S. (1982). Regulation of glucocorticoid receptors in brain by corticosterone treatment of adrenalectomized rats. Neuroendocrinology *35*, 411-417.

Tritos,N., Kitraki,E., Philippidis,H., and Stylianopoulou,F. (1999). Neurotransmitter modulation of glucocorticoid receptor mRNA levels in the rat hippocampus. Neuroendocrinology *69*, 324-330.

Tronche,F., Kellendonk,C., Kretz,O., Gass,P., Anlag,K., Orban,P.C., Bock,R., Klein,R., and Schutz,G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature Genetics *23*, 99-103.

Tronche,F., Kellendonk,C., Reichardt,H.M., and Schutz,G. (1998). Genetic dissection of glucocorticoid receptor function in mice. Current Opinion in Genetics & Development *8*, 532-538.

Truss,M., Bartsch,J., Schelbert,A., Hache,R.J., and Beato,M. (1995). Hormone induces binding of receptors and transcription factors to a rearranged nucleosome on the MMTV promoter in vivo. Embo Journal *14* , 1737-1751.

Truss,M., Chalepakis,G., Pina,B., Barettino,D., Bruggemeier,U., Kalff,M., Slater,E.P., and Beato,M. (1992). Transcriptional control by steroid-hormones. Journal of Steroid Biochemistry and Molecular Biology *41*, 241-248.

Tsuneishi,S., Takada,S., Motoike,T., Ohashi,T., Sano,K., and Nakamura,H. (1991). Effects of dexamethasone on the expression of myelin basic protein, proteolipid protein, and glial fibrillary acidic protein genes in developing rat brain. Brain Research.Developmental Brain Research *61*, 117-123.

Turnell,R.W. and Burton,A.F. (1975). Glucocorticoid receptors and lymphocytolysis in normal and neoplastic lymphocytes. Molecular and Cellular Biochemistry *9*, 175-189.

Turner,B.B. (1992). Sex differences in the binding of type I and type II corticosteroid receptors in rat hippocampus. Brain Research *581*, 229-236.

Uberbacher,E.C. and Mural,R.J. (1991). Locating protein-coding regions in human DNA sequences by a multiple sensor-neural network approach. Proc Natl Acad Sci U S A *88*, 11261-11265.

Vacchio,M.S. and Ashwell,J.D. (1997). Thymus-derived glucocorticoids regulate antigen-specific positive selection. Journal of Experimental Medicine *185*, 2033-2038.

Vacchio,M.S., Papadopoulos,V., and Ashwell,J.D. (1994). Steroid-production in the thymus - implications for thymocyte selection. Journal of Experimental Medicine *179*, 1835-1846.

Valdez,R., Athens,M.A., Thompson,G.H., Bradshaw,B.S., and Stern,M.P. (1994). Birthweight and adult health outcomes in a biethnic population in the USA. Diabetologia *37*, 624-631.

Vallee,M., Mayo,W., Dellu,F., Le Moal,M., Simon,H., and Maccari,S. (1997). Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. J Neurosci *17*, 2626-2636.

van Dijk,M.A., Rodenburg,R.J., Holthuizen,P., and Sussenbach,J.S. (1992). The liver-specific promoter of the human insulin-like growth factor II gene is activated by CCAAT/enhancer binding protein (C/EBP). Nucleic Acids Research *20*, 3099-3104.

van Haarst,A.D., Oitzl,M.S., and De,K. (1997). Facilitation of feedback inhibition through blockade of glucocorticoid receptors in the hippocampus. Neurochemical Research *22*, 1323-1328.

Vanderbilt,J.N., Miesfeld,R., Maler,B.A., and Yamamoto,K.R. (1987). Intracellular receptor concentration limits glucocorticoid- dependent enhancer activity. Molecular Endocrinology *1*, 68-74.

Vazquez,D.M., Lopez,J.F., Morano,M.I., Kwak,S.P., Watson,S.J., and Akil,H. (1998). alpha, beta, and gamma mineralocorticoid receptor messenger ribonucleic acid splice variants: Differential expression and rapid regulation in the developing hippocampus. Endocrinology *139*, 3165-3177.

Vedder,H., Weiss,I., Holsboer,F., and Reul,J.M. (1993). Glucocorticoid and mineralocorticoid receptors in rat neocortical and hippocampal brain cells in culture: characterization and regulatory studies. Brain Research *605*, 18-24.

Vicente,A., Varas,A., Acedon,R.S., Jimenez,E., Munoz,J.J., and Zapata,A.G. (1998). Appearance and maturation of T-cell subsets during rat thymus ontogeny. Developmental Immunology *5*, 319-331.

Vig,E., Barrett,T.J., and Vedeckis,W.V. (1994). Coordinate regulation of glucocorticoid receptor and c-jun mRNA levels: evidence for cross-talk between two signaling pathways' at the transcriptional level. Molecular Endocrinology *8*, 1336-1346.

Wadekar,S.A., Li,D., Periyasamy,S., and Sanchez,E.R. (2001). Inhibition of heat shock transcription factor by GR. Molecular Endocrinology *15*, 1396-1410.

Wallace,A.D. and Cidlowski,J.A. (2001). Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem *276*, 42714-42721.

Watkins,L.R., Wiertelak,E.P., Goehler,L.E., Mooney,H., Martinez,J., Furness,L., Smith,K.P., and Maier,S.F. (1994). Neurocircuitry of illness-induced hyperalgesia. Brain Research *639*, 283-299.

Weaver,I.C., La Plante,P., Weaver,S., Parent,A., Sharma,S., Diorio,J., Chapman,K.E., Seckl,J.R., Szyf,M., and Meaney,M.J. (2001). Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. Mol Cell Endocrinol *185*, 205-218.

Webster,J.C., Jewell,C.M., Bodwell,J.E., Munck,A., Sar,M., and Cidlowski,J.A. (1997). Mouse glucocorticoid receptor phosphorylation status influences multiple functions of the receptor protein. Journal of Biological Chemistry *272*, 9287-9293.

Webster,M., O'Grady,J., Orthmann,J., and Shannon Weickert C (2000). Decreased glucocorticoid receptor mRNA levels in individuals with depression, bipolar disorder and schizophrenia. Schizophrenia Research *41*, 411.

Weidenfeld,J., Itzik,A., and Feldman,S. (1997). Effect of glucocorticoids on the adrenocortical axis responses to electrical stimulation of the amygdala and the ventral noradrenergic bundle. Brain Research *754*, 187-194.

Weis,L. and Reinberg,D. (1992). Transcription by RNA polymerase-II - initiator-directed formation of transcription-competent complexes. Faseb Journal *6*, 3300-3309.

Welberg,L.A., Seckl,J.R., and Holmes,M.C. (2000). Inhibition of 11beta-hydroxysteroid dehydrogenase, the foeto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. European Journal of Neuroscience *12*, 1047-1054.

Welberg,L.A., Seckl,J.R., and Holmes,M.C. (2001). Prenatal glucocorticoid programming of brain corticosteroid receptors and corticotrophin-releasing hormone: possible implications for behaviour. Neuroscience *104*, 71-79.

Wiegers,G.J., Knoflach,M., Bock,G., Niederegger,H., Dietrich,H., Falus,A., Boyd,R., and Wick,G. (2001). CD4(+)CD8(+)TCR(low) thymocytes express low levels of glucocorticoid receptors while being sensitive to glucocorticoid-induced apoptosis. European Journal of Immunology *31*, 2293-2301.

Woolley,C.S., Gould,E., and McEwen,B.S. (1990). Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Research *531*, 225-231.

Woolley,C.S., Gould,E., Sakai,R.R., Spencer,R.L., and McEwen,B.S. (1991). Effects of aldosterone or RU28362 treatment on adrenalectomy-induced cell death in the dentate gyrus of the adult rat. Brain Research *554*, 312-315.

Wu,J. and Grunstein,M. (2000). 25 years after the nucleosome model: chromatin modifications. Trends in Biochemical Sciences *25*, 619-623.

Wu,R.L. and Barish,M.E. (1994). Astroglial modulation of transient potassium current development in cultured mouse hippocampal neurons. Journal of Neuroscience *14*, 1677-1687.

Xue,Y., Murdjeva,M., Okret,S., McConkey,D., Kiuossis,D., and Jondal,M. (1996). Inhibition of I-Ad-, but not Db-restricted peptide-induced thymic apoptosis by glucocorticoid receptor antagonist RU486 in T cell receptor transgenic mice. European Journal of Immunology *26*, 428-434.

Yau,J.L., Morris,R.G., and Seckl,J.R. (1994). Hippocampal corticosteroid receptor mRNA expression and spatial learning in the aged Wistar rat. Brain Research *657*, 59-64.

Yau,J.L., Noble,J., Hibberd,C., and Seckl,J.R. (2001a). Short-term administration of fluoxetine and venlafaxine decreases corticosteroid receptor mRNA expression in the rat hippocampus. Neuroscience Letters *306*, 161-164.

Yau,J.L., Noble,J., Kenyon,C.J., Hibberd,C., Kotelevtsev,Y., Mullins,J.J., and Seckl,J.R. (2001b). Lack of tissue glucocorticoid reactivation in 11beta - hydroxysteroid dehydrogenase type 1 knockout mice ameliorates age-related learning impairments. Proceedings of the National Academy of Sciences of the United States of America *98*, 4716-4721.

Yau,J.L., Noble,J., and Seckl,J.R. (2001c). Acute restraint stress increases 5-HT7 receptor mRNA expression in the rat hippocampus. Neuroscience Letters *309*, 141-144.

Yau,J.L., Noble,J., Widdowson,J., and Seckl,J.R. (1997a). Impact of adrenalectomy on 5-HT6 and 5-HT7 receptor gene expression in the rat hippocampus. Brain Research.Molecular Brain Research *45*, 182-186.

Yau,J.L.W., Noble,J., and Seckl,J.R. (1997b). Site-specific regulation of corticosteroid and serotonin receptor subtype gene expression in the rat hippocampus following 3,4-methylenedioxymethamphetamine: role of corticosterone and serotonin. Neuroscience *78*, 111-121.

Yourick,J.J. and Beuving,L.J. (1985). The effects of insulin on hepatic glucocorticoid receptor content in the diabetic rat. Journal of Receptor Research *5*, 381-395.

Yu,L., Romero,D.G., Gomez,S., and Gomez,S. (2002). Steroidogenic enzyme gene expression in the human brain. Mol Cell Endocrinol *190*, 9-17.

Yudt,M.R. and Cidlowski,J.A. (2001). Molecular identification and characterization of a and b forms of the glucocorticoid receptor. Molecular Endocrinology *15*, 1093-1103.

Zacharchuk,C.M., Merçep,M., Chakraborti,P.K., Simons,S.S., and Ashwell,J.D. (1990). Programmed T lymphocyte death. Cell activation- and steroid-induced pathways are mutually antagonistic. Journal of Immunology *145*, 4037-4045.

Zawel,L. and Reinberg,D. (1995). Common themes in assembly and function of eukaryotic transcription complexes. Annual Review of Biochemistry *64*, 533-561.

Zennaro,M.C., Keightley,M.C., Kotelevtsev,Y., Conway,G.S., Soubrier,F., and Fuller,P.J. (1995). Human mineralocorticoid receptor genomic structure and identification of expressed isoforms. Journal of Biological Chemistry *270*, 21016-21020.

Zennaro,M.C., LeMenuet,D., and Lombes,M. (1996). Characterization of the human mineralocorticoid receptor gene 5'-regulatory region: Evidence for differential hormonal regulation of two alternative promoters via nonclassical mechanisms. Molecular Endocrinology *10*, 1549-1560.

Zhou,D.F., Shen,Y.C., Shu,L.N., and Lo,H.C. (1987). Dexamethasone suppression test and urinary MHPG X SO4 determination in depressive disorders. Biol Psychiatry *22*, 883-891.

Zhou,M.Y., del Carmen,V., Gomez,S., and Gomez,S. (1997). Cloning of two alternatively spliced 21-hydroxylase CDNAs from rat adrenal. Journal of Steroid Biochemistry and Molecular Biology *62*, 277-286.

Zong,J., Ashraf,J., and Thompson,E.B. (1990). The promoter and 1st, untranslated exon of the human glucocorticoid receptor gene are GC rich but lack consensus glucocorticoid receptor element sites. Molecular and Cellular Biology *10*, 5580-5585.